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JAMES PETER HILL

THE
JAMES PETER HILL VOLUME
OF
THE JOURNAL OF ANATOMY

VOLUME 82 OF THE *JOURNAL OF ANATOMY*
IS DEDICATED TO
PROFESSOR J P HILL

J P HILL

D Sc (LOND), Sc D (DUBLIN), (BELFAST), F Z S , F R S

A BIOGRAPHICAL SKETCH OF HIS CAREER AND AN
APPRECIATION OF HIS WORK, TOGETHER WITH
'A BIBLIOGRAPHY OF HIS PUBLISHED WRITINGS

James Peter Hill was born in Kennoway, Fifeshire, on 21 February 1873 His father was one of those great farmers who made Scots agriculture the most productive and advanced in the world, the late Sir John Gilmour (Home Secretary) told me that he was widely known as a judge of horses and as a man whose advice was sought and accepted Hill went as a boy to the Royal High School in Edinburgh and passed on from there to the Royal College of Science in London, where he was a student of G B Howes, who described the development of the skull of *Sphenodon* He then returned to the University of Edinburgh, where Cossor Ewart was Professor of Zoology

In 1892 he was appointed a demonstrator in biology in the University of Sydney, and immediately began to collect and to make preparations of the marine animals to be found in great variety and abundance in Sydney Harbour These preparations, very many of which still exist, show that Hill had already developed that superb technique which has always characterized him Here he soon found an Enteropneustan which he described in 1894 as *Ptychodera australiensis*, subsequently publishing a very detailed account illustrated by beautiful drawings of a further *Ptychodera* Fortunately, J P (as he was affectionately known to his colleagues and students in University College) became associated with J T Wilson, Professor of Anatomy, and with C J Martin, Demonstrator in Physiology, who were actively working in Sydney on the anatomy of the Monotremes, and with G Elliot Smith, then beginning that work which revolutionized our understanding of the morphology of the brain

Thus Hill turned to the study of vertebrates and, with Martin, described a flat embryo of *Echidna*, which provided a link between sauropsidan and mammalian development A little later he showed that *Perameles* possessed an allantoic placenta, and he thereby began an active discussion of the relationships of Marsupials This work, alone or in association with J T Wilson, has continued to the present day Sydney recognized Hill's distinction in this field by appointing him Lecturer in Embryology in 1904, and in 1906 he was appointed, in face of a very distinguished field of applicants, to the Jodrell Chair of Zoology and Comparative Anatomy in University College, London Hill retained this post until in 1921, as part of the development of the Department of Anatomy in the College (made possible by the acceptance by the Rockefeller Trustees of a scheme designed by Elliot Smith), a Chair of Embryology and Histology was founded expressly for him This was intended to be a research post, involving very little teaching but Hill devoted himself

with enthusiasm to the proper teaching of histology as well as that of embryology. Indeed he reached an entirely new standard in this respect, the preparations which were distributed to all students were of such quality that Boecke, the great Dutch neuro-histologist, once said to me that it was improper to give them to medical students, 'never again in their life will they ever see such beautiful things'. This is most characteristic of Hill, he is the most conscientious of men, everything he does is done with the most meticulous accuracy, no one to my knowledge has ever collected, preserved and studied his materials with such precision. And what collections they are! In Australia Hill himself shot and dissected innumerable Monotremes and Marsupials, going out to the forests and streams, often with Wilson and Elliot Smith. He bred *Dasypus* in captivity and thus obtained materials on which many years later the reproductive cycle was worked out. He collected *Didelphys* embryos and those of many other beasts in Brazil. He built up great collections of cat and rabbit, of other rodents and sparrows, and from his former students obtained the materials on which his great work on the placenta was based.

His quality is shown by his research students, he was the best trainer of such men I have ever met. Each was given his problem and material, was shown how to proceed, was visited every day and had his work discussed with him man to man. Nothing must be put down without a full discussion of possibilities. How often have I heard J P disputing whether a particular cell should be included in the neural plate of a flat embryo being graphically reconstructed, or not. Then when the work was complete and written up the exact wording of each sentence had to be discussed to make certain that it was precise, unambiguous, and made all possible reservations. And the illustrations, at first made with a 4H pencil on Bristol Board, with photographs as supporting evidence, received equally careful attention, becoming better as one of his own papers succeeded another until they reached the superb character of Hill's most recent work. So the work went on, very slowly it is true, but of splendid quality, very few men have produced so vast a mass of detailed descriptions containing so few errors of fact. Reference to such a text-book as Brachet's will show how large is the contribution made by Hill and his school to descriptive vertebrate embryology.

But Hill's interests and knowledge extend far beyond embryology, he was the first zoologist to recognize the importance to morphology of Robert Broom's palaeontological work, and introduced his results into undergraduate teaching long before any other zoological department, since the days of Lankester, paid any attention to fossils. He became interested in the study of cytoplasmic inclusions and added Brontë Gatenby to his staff. His undergraduate teaching was balanced and remarkable, invertebrates were dealt with as carefully and completely as vertebrates and general problems received due attention, his old students recall with admiration the wonderful dissections of Molluscs he made when demonstrating to senior students. Embryology he taught as a 'special subject' in a degree course as a deliberate introduction to research

His course was full of detail, discussed with full reference to all the original literature, and it was effective and has no present day parallel. Nearly all the men and women who took a zoology degree in the years before the 1914 war stayed on (despite the lack of research grants) and published work on embryology, a great testimony to the respect and affection he gained from them.

Hill's influence has spread far beyond his own students and associates. He is known personally to all the serious embryologists in the world and has done much to keep up an active friendship amongst them, and in our own country he has done much to bring about a better appreciation of the work of anatomists amongst zoologists, and vice versa. We recognize in J P Hill not only a great master of embryology, but also one of those general zoologists of wide interests and encyclopaedic knowledge who are now so rare, and unreplaced. And those who have the privilege of his friendship know in him a man of delightful and unique personality.

D M S W

It may be asserted without danger of exaggeration that J P Hill has not only laid the foundations of our knowledge of the development of Monotremes and Marsupials, but that he has, by his own researches and those which he has stimulated others to undertake, erected thereon a lasting monument of exact knowledge which constitutes a contribution to science of permanent and inestimable value.

In a series of papers extending over half a century he has unravelled the secrets of the development of the Platypus. At the time when he began to study this material, knowledge was practically restricted to the facts that Monotremes were oviparous and that their eggs were meroblastic. With C J Martin, Hill described the structure of the embryo from the intra-uterine egg, and showed by comparison with Sauropsida and with higher Mammalia that a knowledge of the conditions in the Platypus are essential for the comprehension of many features of vertebrate embryology. With J T Wilson, Hill contributed a detailed account of the organogeny of the Platypus, with T Thomson Flynn he has studied and described in minute detail the processes of growth of the ovarian ovum, its maturation, fertilization, cleavage, and the formation of the germ-layers, with J Bronte Gatenby he has described the formation of the corpus luteum and correlated its phases with the periods of intra-uterine development, of incubation, and of lactation which are passed through in the ontogeny of this remarkable animal. While his daughter, Dr C J Hill, has studied the histology of the oviduct during gestation, Hill has described the structure of the egg-shell and shown how, in early stages, its structure is such as to permit passage of fluid substances from the uterine glands to the embryo, before the deposition of the impermeable protective layer through which pore-canalae enable the respiratory exchange to take place during the period of incubation.

Hill's contributions to marsupial embryology have been no less numerous and fundamental. He demonstrated that parturition in *Perameles* takes place

through the median vaginal canal, and showed the morphological relations of this structure to the oviducts. He discovered that *Perameles* possesses an allantoic placenta—a fact of fundamental importance in the phylogenetic history of the placenta. And in *Dasyurus* he showed that the egg has a layer of albumen and a shell-membrane, that it contains yolk which is detached as a yolk-body before the completion of the first cleavage-division, and that the eggs are produced in large numbers. All these features are evidence of the primitive nature of the Marsupials and are relics of a former ancestral oviparous condition. Hill is at present at work with de Beer on another similar feature—vestiges of the egg-tooth papilla and associated structures in various Marsupials.

With E. A. Fraser, Hill investigated the organogeny of the thyroid, thymus, and epithelial bodies in Marsupials. Turning to Placentals, Hill made, with G. S. Sansom, a careful study of the mode of implantation of the blastocyst of *Cavia*, and showed that the so-called 'inversion of the germ-layers' is brought about by different means in different types. With M. Tribe, Hill described the earliest stages in the development of the cat, in which they were successful in making out a cell-lineage as far as the 63-cell stage.

Early stages in the development of the human embryo have been studied by Hill and J. Florian, with particular attention to the head-process and prochordal plate. Lastly, in his Croonian Lecture, Hill has traced the developmental history of the Primates as evidenced by the constitution of the blastocyst, the type of implantation, the origin and mode of formation of the mesoderm, the formation of the amnion, and the structure of the placenta, through the lemuroid, tarsuroid, plathecoid and anthropoid stages.

All anatomists and zoologists will eagerly await the publication of the work on the neural crests on which Hill is now engaged.

In addition to those whose names have already been mentioned as working with Hill, no account of his contribution to science would be complete without reference to those who, as his pupils or colleagues, have been stimulated by him to undertake researches published under their own names. These include

D. M. S. WATSON	W. ROWAN	E. FAWCETT
K. PARKER	F. THOMAS	R. H. BURNE
R. LUDFORD	N. GOORMAGHTIGH	R. DEANSLEY
C. H. O'DONOGHUE	N. KULCHITSKY	W. F. BATTY GRIFFITH
F. INCE	W. F. ROGERS BRAMBELL	H. B. FANTHAM
A. SUBBA RAU	R. ASSHETON	E. T. BROWNE
K. C. RICHARDSON	J. H. ASHWORTH	D. SIMMONS
P. MACGREGOR	F. H. EDGEWORTH	W. J. DAKIN
J. THORNTON CARTER	J. H. WOODGER	U. FIELDING
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A. GIRGIS	H. H. WOOLLARD	A. A. TARKHIAN
M. BOYD	H. A. HARRIS	Y. H. AASAR
E. C. AMOROSO	W. E. LE GROS CLARK	F. P. REAGAN

G. R. DE B.

The photograph is by Mr F. J. Pittock, F.R.P.S., Department of Anatomy and Embryology, University College, London.

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THE SYNOVIAL JOINTS OF THE SKATE (*RAIA*)

By D V DAVIES, *Anatomy School, University of Cambridge*

Bland Sutton (1903) classifies loose bodies in joints as (a) physiological, and (b) pathological. Of the former he states, 'A good physiological type of loose cartilaginous bodies which infests joints is furnished by the temporo-mandibular joint of the skate. A recess communicating with the articular cavity usually contains a collection of smooth cartilaginous bodies in contour and size like melon seeds'. Interest in both these categories of loose bodies has stimulated investigation of this statement. No other reference to them in the skate nor to a physiological type of loose body in any other animal has been found in the literature.

All the large synovial joints in the skate have been examined, but only the jaw joint contains loose bodies. The joints described here are

- (a) palatoquadrate-mandibular,
- (b) occipito-vertebral,
- (c) those at the base of the pectoral fin, between the pro-, meso- and metapterygia and the pectoral girdle,
- (d) basal joints of the pelvic fin, i.e. of the propterygium (anterior basal cartilage) with the basipterygium and with the pelvic girdle.

The only synovial joint related to the jaws is that between the palato-ptyerygo-quadrate bar and the mandibular (Meckelian) cartilages. It is assumed that this is the temporo-mandibular joint referred to by Bland Sutton. These jaw cartilages are suspended from the skull by a particularly well-developed hyomandibular cartilage which articulates with the cartilaginous elements at its two extremities by dense fibrous joints (syndesmoses). The hyomandibular cartilage forms the sole suspension of the jaws and allows their protrusion to seize food. The joint between the palato-ptyerygo quadrate bar and the mandibular cartilage will be referred to as the mandibular joint and the palato-ptyerygo-quadrate bar will be called the palatoquadrate for brevity.

MATERIAL AND METHODS

In all forty skates have been examined. Though mainly adult or approaching adult dimensions, a few smaller specimens have been included. The latter have proved much more difficult to procure. The specimens, listed in Table 1, represent eight of the twenty-two species of skate. The majority were examined fresh, a few after fixation in formalin. Sections of the various tissues from the joints were examined histologically after fixation in formalin and staining with haematoxylin and eosin. The loose bodies recovered were measured in physiological saline using a micrometer eyepiece.

OBSERVATIONS

(a) *The mandibular joint*

This joint is perhaps best described as a double ball and socket joint (Pl 1 figs 1, 2) The larger part of the articulation is situated laterally and consists of the ball-shaped posterior and lateral extremity of the palatoquadrate, forming a little less than half a sphere, fitting snugly into a slight socket at the lateral end of the mandibular cartilage, forming roughly a third of a sphere Posterior to the socket on the mandible is the fibrous hyomandibular joint Anterior to this and postero-lateral to the socket is a depression giving attachment to a very strong lateral ligament connecting the mandible to the neck of the palatoquadrate A small fossa on the lateral side of this neck receives the anterior attachment of this ligament On the mandible the fossa for the ligament encroaches on the articular cavity, making its rim deficient here as in the acetabular notch on the mammalian hip bone There is a small non-articular ligamentous area within the lateral part of the socket

On the antero-medial side of the main joint is a second smaller joint continuous with it This again is of the ball and socket variety, but the ball-shaped portion on the mandible is less than a third of a sphere and fits into a shallower socket on the palatoquadrate This second joint is placed medial and anterior to the main part of the joint so that its socket lies on the medial side of the neck of the palatoquadrate, the two portions of the joint being continuous along the medial side of this neck, most of which is covered with articular cartilage Posterior to the lesser portion of the joint and toward the dorsal side of the mandible is a small tubercle giving attachment to a strong ligament which runs forwards on the medial side of the joint to be attached to the palatoquadrate along an area extending from the articular margin medially to the teeth Similarly, arising from a tubercle on the dorsal side of, and just anterior to, the socket on the palatoquadrate is another ligament which passes posteriorly and medially, crossing the first ligament in cruciate fashion on its dorsal side Posteriorly it fans out on to the anterior margin of the mandible from the joint to the teeth The capsule of the joint is thus strengthened medially and laterally by these ligaments, below and, more particularly, above the capsule is lax, forming in these situations two pouches communicating freely with the joint cavity

On opening the joint the synovial membrane presents little of interest apart from the presence in all species of a small number of rather large club-shaped villi projecting into the cavity (Pl 2, figs 11, 12) Histologically they are similar in structure to the synovial membrane lining the joint, and are vascular The construction of the joint surfaces, the arrangement of the ligaments and the appearance of the synovial membrane are similar in all species

Of the eight species examined the loose bodies described by Bland Sutton are found in three only, namely *Rana clavata*, *R. montana* and *R. asterias* In these the loose bodies are constantly present and conversely in the remaining

five species they are always absent (Table 1). This species distribution and the fact that the joint structures show no other obvious species differences suggests that the loose bodies are physiological and not pathological. They are in the main distributed in the looser pockets placed dorsally and ventrally, particularly in the former where they lie behind the neck of the palatoquadrate cartilage, between this and the capsule, which is here attached some distance from the articular margin. These pockets are merely formed by laxity of the capsule and synovial membrane and Bland Sutton's description of 'a recess communicating with the articular cavity' conveys an erroneous impression in so far as it implies a single recess. A few loose bodies, however, are found scattered elsewhere in the joint at random and are frequently seen between the articular surfaces. They occur in the last-named position in fish which have been fixed whole by immersion in formalin and in those stored in the refrigerator and examined before thawing is complete.

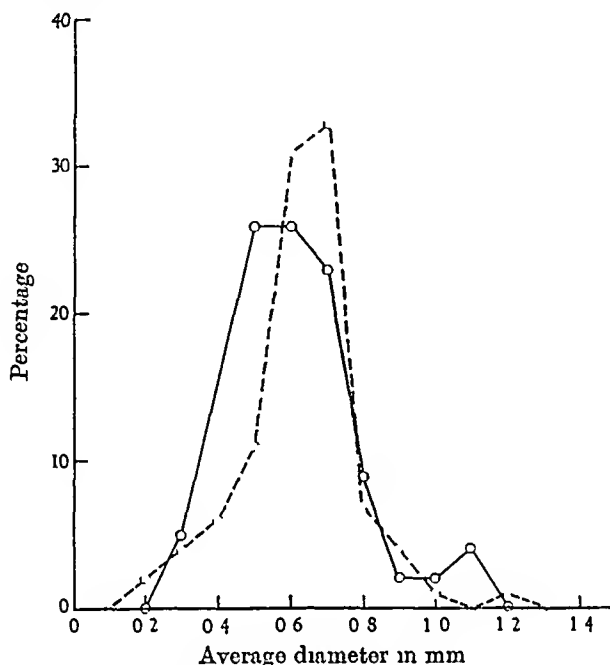
TABLE 1

Species	No. examined	Loose bodies
<i>Raja clavata</i>	18	Present
<i>R. batis</i>	3	Absent
<i>R. radiata</i>	5	Absent
<i>R. circularis</i>	2	Absent
<i>R. montagui</i>	5	Present
<i>R. asterias</i>	3	Present
<i>R. naevus</i>	3	Absent
<i>R. fullonica</i>	1	Absent

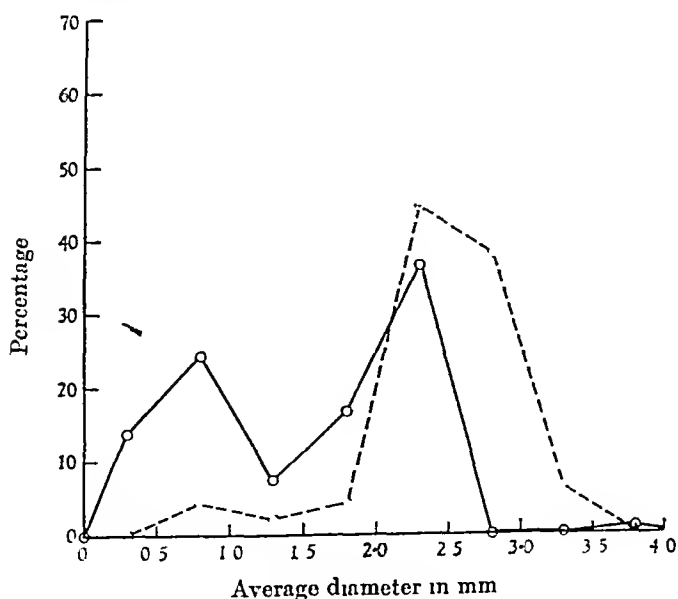
In fish of comparable size the loose bodies in *R. clavata* are very much larger than in either of the other species and have an average diameter in the adult of about 2.0 mm (Pl. 1, figs. 3 and 5). On the other hand, the adult *R. montagui* possesses much smaller bodies, under 1.0 mm in diameter, and *R. asterias* possesses bodies of an intermediate size. The species could be identified in the adult with fair certainty merely from the dimensions of the loose bodies. Graphs 1 and 2 illustrate the differences in size distribution in *R. clavata* and *R. montagui*.

It is difficult to draw many conclusions from this series regarding variations in the number of loose bodies. It appears, however, that the average number in *R. montagui* is considerably higher than in either of the other species, but it is similar in *R. clavata* and *R. asterias* so that the number in any given species does not bear a constant relation to the size of the loose bodies (Table 2). There is a great variation in the number of loose bodies within the species even for fish of comparable size and there is no clear evidence that the number of loose bodies varies in any regular manner with age. This is best seen from Table 2 where the fish are arranged approximately in order of size. The number of loose bodies may differ appreciably, to the extent of 100% or more, in the right and left joints of the same specimen. There is, however, a clear correlation between the size of the fish and the size of the loose bodies contained in its joints.

The range in size of loose bodies obtained from any one joint has proved interesting This can only be satisfactorily studied in the case of the easily obtained *R clavata* An idea of the uniformity of the size distribution in the remaining species is obtained from Graph 1, and Table 3 for *R montagu* This type of graph adequately represents the size distribution in all the *R montagu*



Graph 1 to show size distribution of loose bodies in *R. montagu* No 26
Continuous line = right joint, interrupted line = left joint



Graph 2 to show size distribution of loose bodies in *R. clavata* No 33
Continuous line = right joint, interrupted line = left joint

TABLE 2

R. clavata Adult Total length 85.0 cm Width of disc 61.0 cm
(Blegaud, Faune ichthyologique de l'Atlantique Nord)

Cat no	Total length (cm)	Width of disc (cm)	Inter orbital least width (cm)	Length of snout (cm)	No. of loose bodies		
					Right	Left	Average
2	27.5	18.0	1.0	3.5	101	79	90
36	62.5	56.0	3.0	8.7	111	87	99
28	62.0	45.5	3.2	7.7	146	164	155
14	—	—	3.2	8.5	86	75	80.5
29	67.0	48.0	3.3	8.2	154	206	190
26	72.0	—	3.4	8.6	66	96	81
1	72.5	46.0	—	—	62	50	56
34	—	—	3.8	9.5	119	134	126.5
35	—	—	3.9	9.3	73	83	78
16	—	—	4.0	10.0	98	97	97.5
32	—	—	4.1	10.0	81	75	78
33	—	—	4.3	10.2	95	47	71
6	—	—	4.5	11.7	217	175	196
15	—	—	4.6	11.1	121	132	126.5
25	86.0	—	4.6	11.4	77	96	86.5
31	—	—	5.0	11.4	141	161	151
22	92.5	68.0	5.2	11.5	223	278	250.5
30	—	—	5.9	13.2	107	146	126.5

R. montagui (*R. maculata*) Adult Total length 74.5 cm Width of disc 51.5 cm

12	—	—	2.3	7.1	225	111	168
13	—	—	2.3	7.5	—	254	254
11	—	—	3.0	9.0	265	—	265
24	63	42	2.8	8.0	286	184	235
26	66.3	43.9	2.8	8.8	304	264	284

R. asterias Adult Total length 45.0 cm Width of disc 30.0 cm

21	36.0	24.5	1.4	4.5	130	34	82
17	42.0	29.5	2.0	5.1	119	121	120
18	42.0	30.0	2.0	5.8	187	207	197
20	52.0	35.0	2.2	5.7	—	87	87

TABLE 3 Size distribution of loose bodies

R. montagui No. 26

Right		Left	
Average diameter (mm)	%	Average diameter (mm)	%
0.3	5	0.2	2
0.4	3	0.3	4
0.5	26	0.4	6
0.6	26	0.5	11
0.7	23	0.6	31
0.8	9	0.7	33
0.9	2	0.8	7
1.0	2	0.9	4
1.1	4	1.0	1
—	—	1.1	0
—	—	1.2	1

and *R. asternas* studied. In *R. clavata*, however, though the majority have a similar size distribution curve, a certain number of the larger specimens show the two peaked curve (Graph 2, Table 4) best marked in the case of the right palatoquadrate mandibular joint. In these the majority of the loose bodies are large, but there is also an appreciable number of smaller bodies (Pl 1, fig 6). Co-existing with this, in many of these larger *R. clavata* as well as in one *R. montagu*, there are loose bodies of a shape not unlike the budding yeast cell, a larger parent cell with a smaller rounded projecting daughter cell connected with the main mass by a stalk of variable thickness. The significance of the smaller loose bodies and of this appearance will be discussed later. This appearance could not be produced artificially by pricking or incising the loose bodies.

TABLE 4 *Size distribution of loose bodies*

<i>R. clavata</i> No. 33			
Average diameter (mm)	Midpoints of class intervals	Absolute no Right	%
0.1-0.5	0.3	13	13.7
0.6-1.0	0.8	23	24.2
1.1-1.5	1.3	7	7.4
1.6-2.0	1.8	16	16.8
2.1-2.5	2.3	35	36.8
2.6-3.0	2.8	—	—
3.1-3.5	3.3	—	—
3.6-4.0	3.8	1	1.0
		Total 95	99.9
Left			
0.1-0.5	0.3	0	0
0.6-1.0	0.8	2	4.3
1.1-1.5	1.3	1	2.1
1.6-2.0	1.8	2	4.3
2.1-2.5	2.3	21	44.7
2.6-3.0	2.8	18	38.3
3.1-3.5	3.3	3	6.4
		Total 47	100.1

When examined fresh in saline the majority of loose bodies are ovoid but flattened in one transverse plane (Pl 1, fig 3). Those of *R. clavata* and *R. asternas* are generally uniform in shape, though occasional rounded or much elongated specimens are seen. These latter are more frequent in *R. montagu*. When examined after fixation *in situ* either by formalin or freezing, the majority of the loose bodies are four-sided pyramids due to mutual compression, some are oval discs (Pl 1, fig 4). These latter are found between the articulating surfaces and form good evidence that these bodies do penetrate between these surfaces during life. When floated in saline and particularly when unfixed, the loose bodies, mainly due to their shape, settle quickly and pack very easily and closely on the bottom of the containing vessel.

The physical characters of the loose bodies are best described as hard but elastic. When placed between two glass slides they slip with ease and generally

escape. It is difficult to squash them and to make a smear of their constituent tissue. One smear is shown in Pl 2, fig 10.

On section (Pl 2, figs 7-9) the loose bodies from all species consist of living cells with a large quantity of fibrillar intercellular substance. In the central part of the loose body this latter is more compact and stains more densely with eosin. In its superficial part the ground substance is less evident. The surface itself is more condensed and smooth and contains few cells. The central part of the ground substance shows an arrangement in concentric spheres or lamellae, this is not seen in the superficial layer. The cells are relatively few. In the central part they are mostly ovoid in shape with a darkly staining nucleus and many cells are surrounded by a clear area separating them from the surrounding ground substance. In the superficial part of the loose body the cells are slightly larger, irregular and provided with long fibrils which pass out into the ground substance and towards the surface, the majority of the fibrils being orientated perpendicular to the surface, as are the parent cells. A few cells occur on the surface but no fibrillar prolongations can be traced from them. Though superficially the tissue resembles cartilage, particularly in its central portion, it is so unlike the other cartilages of the skate that Bland Sutton cannot be right when he maintains that it is cartilage, it must be considered as fibrous tissue in its entirety, the central portion being condensed and possibly not actively growing, whilst the superficial layer is looser and in active growth. Where so-called budding is seen the loose body shows a central condensed nucleus for the larger or parent portion and a second independent condensed centre in the daughter mass (Pl 2, fig 8). This suggests true budding, the only other possibility being malformation of loose bodies in the first place. There is nothing to support this latter view nor is there anything to suggest that this is a degenerative change, such loose bodies presenting no other feature to distinguish them from the more usual type. Nor is there any evidence that small loose bodies have arisen by a degenerative change in larger ones. Both types present a similar structure.

Synovial fluid occurs in the mandibular joint in moderate amount both in the absence and presence of loose bodies. It is clear, colourless and viscid. In amount it compares favourably with that seen in the basal pelvic fin joints and is more abundant here than in either the occipito-vertebral or pectoral fin joints.

(b) The occipito-vertebral joint

The joint between skull and vertebral column is bilateral and surrounded by an extremely strong and tense fibrous capsule. The joint surfaces are almost flat and lie vertically. The joint cavity is narrow and divided into two by a complete fibro cartilaginous disc. This disc is very thin centrally but thickens slightly at the periphery (Pl 3, fig 13). It consists of vertically arranged bundles of fibrous tissue and shows on section a tendency to split in this direction (Pl 3, fig 14). The cartilage cells are very few and scattered in the central portion but more numerous and arranged in vertical rows at the

periphery Like the so-called fibrocartilages in the mammals its peripheral portions only are vascularized A minimal amount of synovial fluid moistens the articular surfaces Unlike all other specimens examined, one *R. montagu* shows central perforation of both discs This is probably a variation, similar variations are well known in man

(c) *Joints of the pectoral fin*

The joints at the base of the pectoral fin between the pro-, meso- and metapterygia and the pectoral girdle are all shallow ball and socket types of synovial joint, the socket being placed in each case on the lateral element The joint between the mesopterygium and the girdle is the largest and is placed some $\frac{1}{2}$ –1 cm closer to the median plane than the joints on either side of it The joints between the pro- and metapterygia are approximately equal in size Each joint contains just enough synovial fluid to moisten the articular surfaces The capsular ligaments are short and thick so that the fit between the elements of the joint is a very close one The synovial membrane is small in extent and relatively smooth showing no villi or folds as in the pelvic fin joints

(d) *Joints of the pelvic fin*

The joint between the basipterygium and the pelvic girdle is similar to the basal joints of the pectoral girdle

The joint between the propterygium and the girdle presents several interesting features Of the two surfaces, that on the pelvic girdle forms a narrow arc, convex in all directions and about a quarter of a circle in extent (Pl 3, fig 15) On the propterygium the articular surface forms a curved trough, the long axis of which makes an arc of about one-sixth of a circle This articular surface is concave in all directions Thus the joint surfaces here are quite unusual This type of joint allows very free range of movement in the direction of its long axis and an appreciable range in a plane at right angles to this

The capsule of this joint is relatively thin and lax The interior of the joint shows a curious cobweb-like reticulum occupying all the recesses of the joint and best developed in the gutters on the medial and lateral side (Pl 3, fig 16) Attached to the reticulum are numerous irregularly scattered white nodules On section the reticulum is seen to arise from the synovial membrane and consists of very fine strands, both cellular and acellular, running across the recesses and projecting freely into the joint (Pl 3, fig 17) The white nodules on the reticulum consist of masses of connective tissue cells (Pl 3, fig 18) No blood vessels are seen either on the reticulum or the attached nodules In addition to the above, club-shaped villi, such as those in the mandibular joint, are frequently seen arising from the synovial membrane

DISCUSSION

The unique feature in the synovial joints examined is the presence of loose bodies in the mandibular joint, as previously noted by Bland Sutton. There is little to suggest that these are pathological. Their constancy in certain species at all ages and their absence in others negative such a suggestion. Furthermore, the other joint structures are similar in all species irrespective of the presence of loose bodies and neither cartilage nor synovial membrane show any pathological change.

Their function is, however, perplexing. The first and most obvious suggestion is that they act as ball bearings. These latter have two distinct actions: (a) they convert sliding friction to rolling friction which as a rule is much the less, thus they diminish the friction between the joint surfaces in movement, and (b) they diminish the starting friction under a load. In a sliding joint the lubricant is squeezed out when resting. In the mammals the conditions and nature of the synovial fluid on the whole makes such squeezing out unlikely.

As already noted, synovial fluid is present in appreciable quantity in the mandibular joint. The shape of the loose bodies (ovoid) and their relative softness are not in favour of any action such as that described in (a). They are sufficiently soft to be readily deformed and flattened when interposed between the articular surfaces. From a comparison with other animals it does not seem that the second function of ball bearings described above is performed by these bodies. In the absence of any method of packing these loose bodies and of maintaining their relative positions, such as in a ball race, it is difficult to see how they can act as efficient ball bearings.

The function of filling dead space within the joint cavity in its different positions and movements seems more likely. These bodies glide on one another and on other surfaces with great ease. They are elastic and easily distorted. When floated in fluid they quickly collect at the bottom of the receptacle and in so doing pack neatly and closely on account of their shape and slipperiness. In some of the larger *R. clavata* examined the loose bodies from one joint, when packed in this manner, occupy a volume of as much as 1 c.c. In the largest *R. montagu*, however, their total volume is only a fraction of this, and they are more like sand than ball bearings. From an examination of the joint surfaces it seems improbable that both the large and small portions of the joint surface remain in contact in the extremes of movement. It seems likely that one or other gets removed from its twin surface, in which case the loose bodies might fill the void, and, on account of their slipperiness and shape, be readily squeezed out again when the surfaces re-engage. The only difficulty in accepting this explanation is that the mechanical conditions at the articulating surfaces are the same in all species, but loose bodies are present in only a few. Those without loose bodies present no obvious compensatory mechanism. The fact that the loose bodies grow with their host would agree with this type of function as the joints and their volume must also grow proportionately.

However, the movements at the joint surfaces in the living fish will have to be observed before such a view can be substantiated. In any case if this were the sole function it would appear that the disadvantages of such a mechanical arrangement would far outweigh the advantages.

There is nothing to suggest that the loose bodies are derived from the detritus cast into the joint during the wear and tear of movement, nor that they are in any way involved in the removal of such detritus. In the present state of our knowledge of the origin of the synovial fluid it would be unwise to exclude a function associated with this for the loose bodies.

In the larger skates the presence of some very small loose bodies associated with many large ones suggests degeneration, failure of growth or production of new loose bodies. There is no evidence that these are degenerate, nor is there anything in their structure to suggest a failure or retardation of growth. If the synovial fluid or synovial membrane from which these bodies must ultimately derive all their nourishment were insufficient to provide for their maintenance or growth, it would be difficult to believe that the incidence of this impoverishment would fall entirely on a few bodies to the exclusion of others. The occasional co-existence of large and small loose bodies suggests that the latter may be derived by active 'division' in response to a stimulus in this manner. The origin of crops of loose bodies from the synovial membrane in adult life is a possibility, but on section nothing is seen in adult membrane to suggest this view. The initial source of all these bodies in the young skate must be the synovial membrane or the mesenchyme occupying the site of the future joint cavity at the time of cavitation to form a synovial joint. It might even be that the cobweb-like reticulum in the propterygium-pelvic girdle joint is an arrest in an earlier stage of the process and that the future loose bodies are represented by the nodules.

In the anterior pelvic joint the reticulum undoubtedly represents a failure of complete cavitation or rather an extension of cavitation in an incomplete state to the tissues around the original joint. The type of joint here favours a very wide excursion of the pelvic fin in one direction and such loose tissue around the joint would be of great advantage. In addition this reticulum would facilitate the passage of substances in and out of the joint and owing to its irregular cobweb nature would help to keep the more central part of the joint clear of detritus even though devoid of living cells in many places.

Articular discs have been described previously in fish by Sullivan (1922) and Petersen (1914) and it would seem that if more were known of their distribution their incidence might be found to be higher in fish than in mammals. The disc in the occipito-vertebral joint of the skate is biconcave though not markedly so. Thus, placed in an almost plane-gliding joint, supports the view that it acts as a Michel pad as suggested by MacConaill (1932). In an animal whose skeleton is mainly uncalcified and partly calcified cartilage it does not seem likely that such thin intra-articular discs have a shock-absorbing function, nor do they compensate for any incongruities in the joint surfaces.

SUMMARY

- 1 The larger synovial joints of the skate (*Raja*) are described
- 2 Notable features are the presence of loose fibrous bodies in the jaw joint, a fibrocartilaginous disc in the occipito-vertebral joint and a reticulum occupying the recesses of the propterygium-pelvic girdle joint
- 3 The distribution, characters, nature and function of the loose bodies and other intra-articular structures are discussed

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EXPLANATION OF PLATES

PLATE 1

- Fig 1 Articular end of Meckel's cartilage of the left side from *R. clavata* No 15. The wide socket, A, receives the head of the palatoquadrate cartilage. Postero-lateral to this is a ligamentous area, B, for the lateral palatoquadrate mandibular ligament. This area is limited on its lateral side by a lip of cartilage. Antero-medial to the main socket is a rounded area, C, articulating with the neck of the palatoquadrate cartilage. $\times 2$
- Fig 2 Posterior end of the left palatoquadrate cartilage from *R. clavata* No 15 showing the rounded bead for articulation with the socket on the mandibular cartilage. Below the head is the shallow socket for the smaller and subsidiary portion of the palatoquadrate mandibular joint. $\times 2$
- Fig 3 A random sample of the loose bodies from the left jaw joint of *R. clavata* No 6 before fixation. $\times 3$
- Fig 4 A random sample of the loose bodies from the right jaw joint of *R. clavata* No 6 after fixation in formalin. The pyramidal shape of the bodies is well seen. $\times 3$
- Fig 5 A random sample of the loose bodies from *R. montagui* No 12 for comparison with those of *R. clavata*. $\times 3$
- Fig 6 A selection of loose bodies from *R. clavata* No 25 showing the range of sizes present and 'budding' of loose bodies. $\times 3$

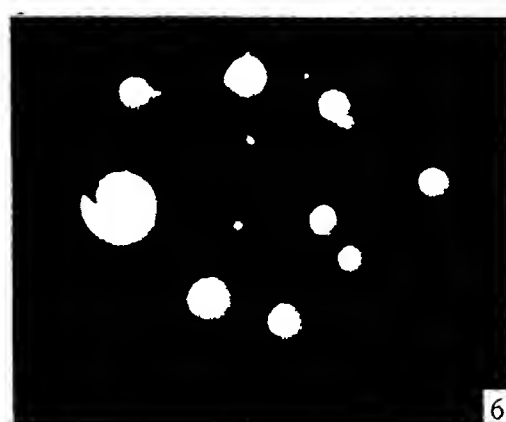
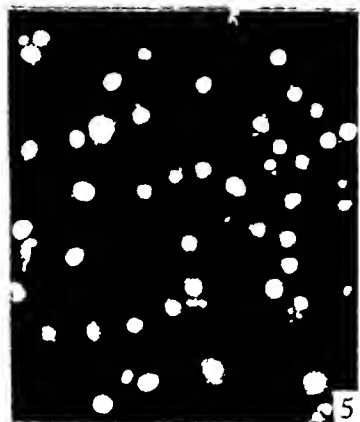
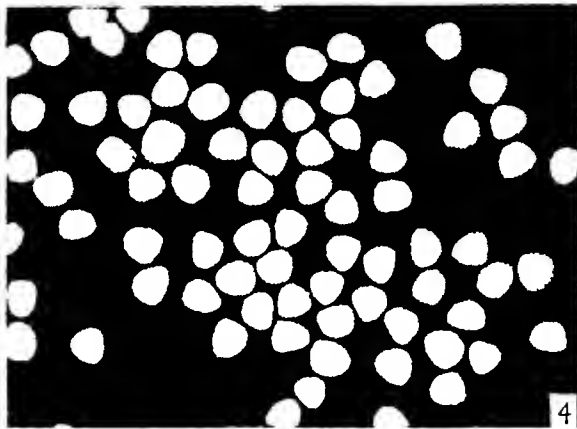
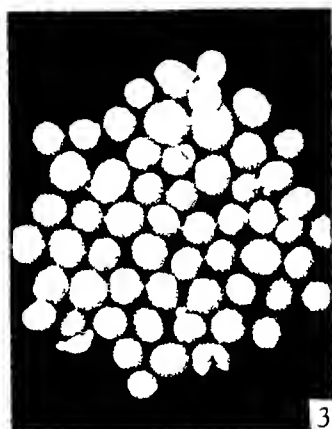
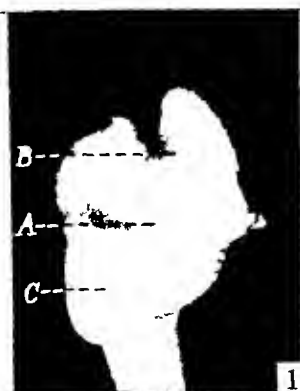
PLATE 2

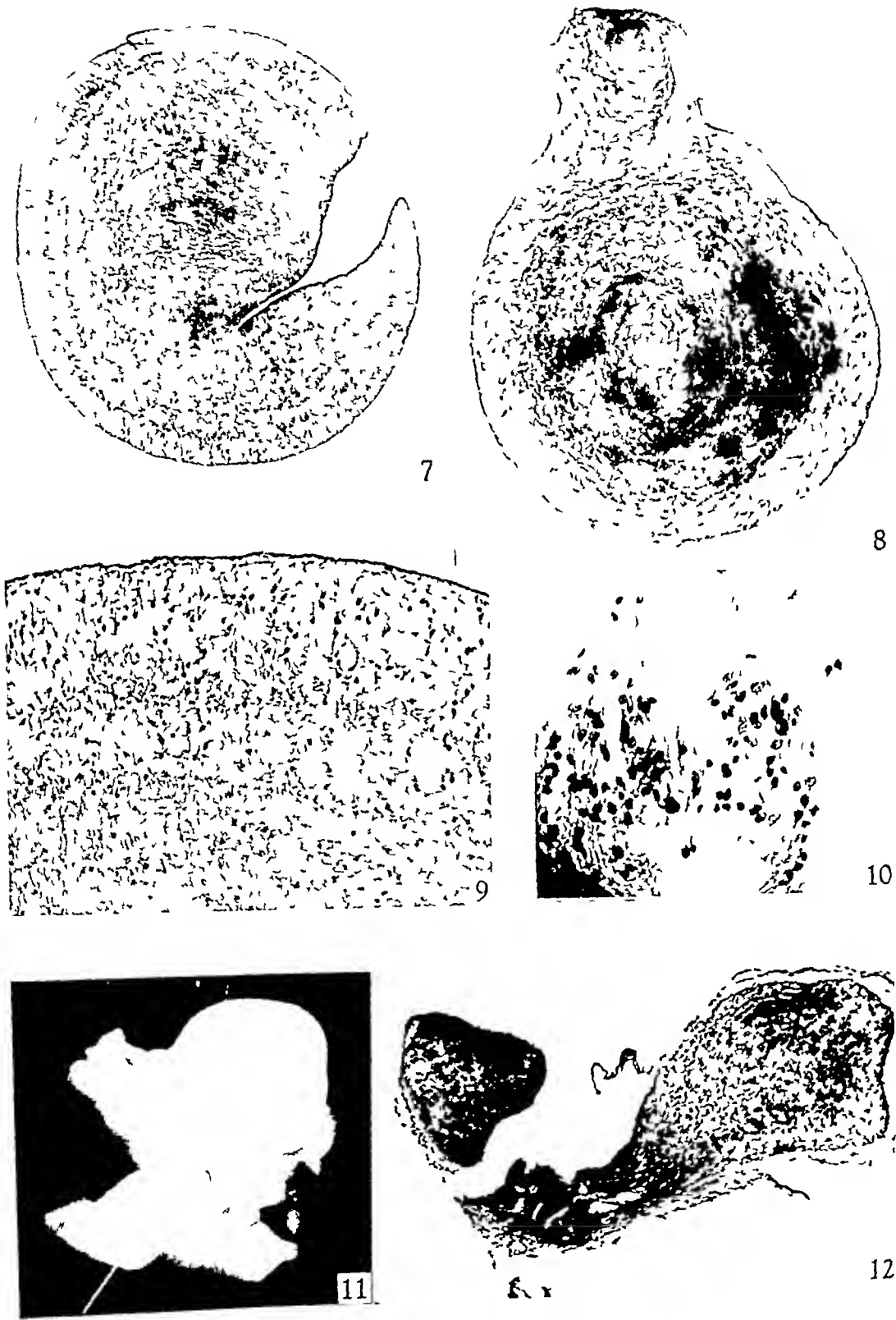
- Fig 7 Section of a large loose body from *R. clavata* No 25. The large fissure is an artifact. Stained with haematoxylin and eosin. $\times 22\frac{1}{2}$
- Fig 8 Section of a loose body from *R. clavata* No 25 showing budding. Stained with haematoxylin and eosin. $\times 43$
- Fig 9 High power view of a portion of the large loose body shown in Fig 7. Stained with haematoxylin and eosin. $\times 98$
- Fig 10 A smear made by squashing a loose body from *R. clavata* between two glass slides. Fixed wet with Bouin's fluid and stained with iron haematoxylin. $\times 280$

- Fig 11 Head and neck of pterygoquadrate cartilage from *R claiata* No 14 showing the club shaped villi
- Fig 12 Sections of the club shaped villi from the synovial membrane of *R claiata* No 14 $\times 45$

PLATE 3

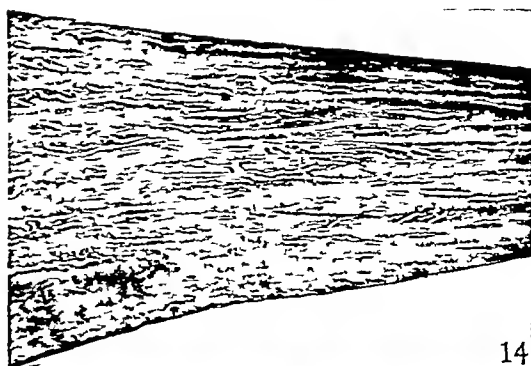
- Fig 13 Intra articular disc from the occipito vertebral joint of an adult *R claiata* $\times 3$
- Fig 14 Section of the intra articular disc from the occipito vertebral joint of *R claiata* $\times 112$
- Fig 15 Joint surface on the pelvic girdle for the propterygium From *R claiata* No 25 Viewed from below $\times 3$
- Fig 16 Portion of the joint surface from the propterygium pelvic girdle joint of *R claiata* No 25 showing the woolly reticulum and the associated nodules which fill the recesses of the joint $\times 3$
- Fig 17 Section of the villi and reticulum in the recesses of the pelvic girdle propterygium joint in *R claiata* No 25 Stained with haematoxylin and eosin $\times 570$
- Fig 18 Section of a recess of the propterygium pelvic fin joint of *R claiata* showing the more cellular nodules in the reticulum The pelvic girdle is on the left side of the photograph Stained with haematoxylin and eosin $\times 22\frac{1}{2}$







13



14



15



16



17



18

THE CHANGES OCCURRING IN THE OVARY OF THE GOAT DURING THE ESTROUS CYCLE AND IN EARLY PREGNANCY

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INTRODUCTION

During the last forty years many papers have been written on the changes occurring in the ovaries of the common domestic ungulates. Yet there is still a gap in our knowledge of the histological changes that occur in the ovary of the goat. Corner (1915, 1919 and 1921) and Solomons & Gatenby (1924) have described the changes that occur in the ovary of the sow, whilst Marshall (1904), Grant (1934) and Warbritton (1934) cover certain aspects of these changes in the ovary of the sheep. The details of some of the changes occurring in the ovary of the cow have been described by McNutt (1924) and all the available literature was summarized by Hammond (1927). The histological changes occurring in the ovary of the mare and the pony have been the subject of recent papers by Hammond & Wodzicki (1941) and Harrison (1946). Kupfer (1928) has given a description of the macroscopic appearances of 20 Boer and 20 Angora goat ovaries from South Africa, but no histological details are presented. The following work was therefore undertaken to investigate oogenesis, follicular development and atresia, and the development and fate of the corpus luteum in the ovary of the goat.

MATERIALS AND METHODS

The ovaries on which this report is based can be divided into four groups: first, the ovaries of twenty-three animals (Table 1) killed a known time after the onset of estrus, secondly, those of 6 goats killed a known time after mating during the first 7 days of pregnancy, thirdly, those of 12 goats killed a known time after mating during the 20th to the 60th day of pregnancy, fourthly, those obtained from 10 goats killed in local slaughterhouses. The ages of the corpora lutea and details of the stage of development of the eggs and embryos recovered from the pregnant animals are given in Table 2. The ovaries of the first five animals in Table 2 were recovered from the animals used by Amoroso, Griffiths & Hamilton (1942) and were made available for this report through the kindness of these authors. The age, stage and location of the eggs recovered from these five animals are fully described in their paper.

The onset of estrus was determined by the observation of the mutual behaviour of the nanny and the billy (Amoroso, Griffiths & Hamilton, 1942). Estrus was dated from the time the female would 'stand' and allow the male to mount to the time of last acceptance of the male. In the case of the non-pregnant series when a particular female was found to associate freely with the male she was removed from the flock and suitable precautions were taken to

TABLE 1 *Non-pregnant goats*

Animal no	Day of cycle killed	Approximate age of corpus luteum
14	1st	Killed 12 hr after onset of full heat Had not ovulated
18	1st	Killed 24 hr after onset of full heat Had not ovulated
28	2nd	6-12 hr
40	2nd	12-18 hr
13	2nd	12-18 hr
26	3rd	18-24 hr
31	3rd	24-30 hr
8	3rd	36-42 hr
39	4th	60-72 hr
25	6th	4-5 days
37	6th	4-5 days
15	9th	7-8 days
36	9th	7-8 days
41	10th	8-9 days
30	12th	10-11 days
38	12th	10-11 days
32	13th	11-12 days
27	16th	14-15 days
33	17th	15-16 days
35	19th	17-18 days
42	19th	17-18 days
34	20th	18-19 days
24	21st	19-20 days

TABLE 2 *Pregnant goats*

Animal no	Time since mating	Remarks
4	30½ hr	Egg located in the tube First cleavage spindle
1	48 hr	Egg located in the tube Second cleavage spindle
5	60 hr	Egg located in the tube Four cell stage
2	85 hr	Egg located in the tube Eight cell stage
7	120 hr	Eggs located in the uterus and tubo uterine junction Thirty two cell stage
6	7 days	Blastocyst present in the uterus
12	20 days	7 mm embryo
11	29 days	13 mm embryo
16	33 days	18 and 20 mm embryos
17	37 days	26 mm embryo
23	38 days	27 and 28 mm embryos
22	39 days	25 mm embryo
9	40 days	31 mm embryo
21	42 days	33 and 34 mm embryos
20	44 days	35 and 36 mm embryos
19	46 days	45 and 45 mm embryos
10	55 days	70 mm embryo
3	60 days	82 mm embryo

prevent mating. Since ovulation is said to occur towards the end of heat, the animals used in the pregnant series were served as late in heat as was possible and killed at the times necessary for recovering eggs of the desired age.

Asdell (1929) states that the duration of estrus in the goat is from 48 to 72 hr, Polocova & Fomenko (quoted by Asdell, 1946) give the duration of estrus as 39.2 ± 1.9 hr, with a spread from 1 to 4 days. In the first series 14 of the 23 goats were under observation for three or more consecutive cycles before being killed, and in these the duration of estrus was from 36 to 48 hr. Ovulation had occurred by the 40th hr after the onset of heat in four animals of the series, but not by the 24th hr in two animals (see Table 1). Thus if ovulation is assumed to have occurred 30–36 hr after the onset of heat, and the age of the corpus luteum timed from that point, the error in timing is probably within ± 12 hr. This error also allows for the few hours' inaccuracy in the determination of the onset of heat. Examination of the recently ruptured follicles and of the early corpora lutea of all the animals used shows that ovulation is spontaneous from all mature follicles, agreeing with the statement of Drummond-Robinson & Asdell (1926).

The goats were obtained from Northern Ireland, and locally from round Glasgow, during the years 1933 to 1947, their breed was indeterminate. Immediately after killing the ovaries were cut in half and fixed in 12% formalin, Zenker-formol, Bouin's or Susa's fluid. Half of the ovaries in the first series were sectioned serially, and representative sections were made of the remainder. The sections were variously stained with Harris's or Heidenhain's haematoxylin, eosin or van Gieson's stain, and with Mallory's triple stain. Occasional sections were stained with Robb Smith's modification of Foot's method, and with osmic acid. Investigations were also made for luteolipin by the methods described by Rossman (1942).

THE OVARY

The ovary is bean-shaped in form, and varies from 2 to 3 cm in length, 1 to 2 cm in depth and $\frac{1}{2}$ to 1 cm in thickness. The weight varies from 11 to 30 g. The ampulla of the oviduct hangs over the lateral and posterior aspect of the ovary, and in many of the specimens examined, especially those killed during estrus and early metestrus, one fimbria was securely attached to the tunica albuginea opposite the hilum of the ovary and close to the impending point of rupture of the mature follicle. The medulla and hilum of the ovary are composed of dense, highly vascular, fibrous tissue. The cortical layer is thin, covering the medulla, except at the hilum. The cortex contains the oocytes, egg nests and the primordial follicles. As the follicles develop they sink down into the medulla. The interstitial tissue is wanting, as in the other ungulates. Under the germinal epithelium is the tough tunica albuginea, which gives the surface of the ovary a smooth appearance. This structure seems to prevent much protrusion of ripe follicles, and herniation of the corpus luteum was only found in a few of the early specimens. The follicles appear to rupture

at any point, except where the ovary is attached to the broad ligament, and there is no ovulation fossa or groove as is found in the mare. In the ovaries under discussion ovulation occurred mainly at the poles of the ovary, up to three follicles rupturing on one side. There was no evidence in the series that one ovary supplied more mature follicles than the other.

OVOGENESIS

Ovogenesis in the goat occurs rhythmically and is definitely related to the estrous cycle. The formation of oocytes is probably a continuous process throughout the cycle, but the maximum number of oocytes is produced during the last 5 days of metestrus and during proestrus. Even during estrus there is a markedly increased number of small healthy oocytes close to the margin of the cortex. The smallest oocyte in the goat ovary measures 20μ in diameter and is surrounded by a few flattened epithelial cells. In many oocytes the subsequent enlargement to 40μ results in the breakdown of this encircling sphere of epithelial cells at points nearest and farthest from the surface of the ovary. Thus most of the granulosa cells first appear at two opposing poles of the oocyte, and subsequently insinuate themselves round the oocyte until they meet and thus form the definitive primordial follicle. This often results in the earliest granulosa cells appearing to form an ellipse about the developing oocyte. Many follicles give evidence of this early polarity of the granulosa cells by their elliptical appearance at a later date in their growth.

The theca interna cells appear as a layer of poorly differentiated small flattened cells when the primordial follicle has a diameter of about 100μ . By the time the developing membrana granulosa has a diameter of 300μ the theca interna has become 6-7 cells thick. Even at this stage there is often a marked 'thecal cone' pointing towards the periphery of the ovary. The thecal cells on the side of the follicle nearest the medulla are flattened and their nuclei are fusiform. The cells lie tangentially about the developing follicle, as if they were being subjected to pressure. The cells of the thecal cone, however, are large, polygonal in shape, and their cytoplasm has a granular 'foamy' appearance. Their nuclei are oval and vesiculated (see Pl 1, fig 6), and mitotic figures are frequently encountered.

The zona pellucida first appears when the oocyte is about 60μ in diameter and the follicle has a diameter of 100μ . By the time the oocyte is 80μ and the follicle 300μ in diameter the zona pellucida is fully developed. In some ovaries there is a precocious development of the oocyte and an associated failure of development of the granulosa and theca interna elements. The diameter of these precocious oocytes is of the order of 75μ . The zona pellucida is present and contained within it are numbers of elongated fusiform bodies that stain blue with haematoxylin. These bodies are possibly the nuclei of granulosa cells absorbed into the zona pellucida. Such bodies have only been found in these precocious oocytes, and their occurrence appears to be restricted to ovaries obtained from animals killed late in estrus or 1 or 2 days after estrus (Pl 1,

fig 5) These observations are presented in support of the theory that the zona pellucida is developed from the granulosa cells. In this connexion the reader is referred to Mainland (1932) for a description of what appear to be similar bodies in the zona pellucida of tubal ova and large ovarian ova of the ferret, and to Guthrie & Jeffers (1938) and Kingsbury (1939) for discussion of the origin of the zona pellucida in the mammalian ovary.

POLYNUCLEAR OVA AND POLYOVULAR FOLLICLES

A striking characteristic of the goat ovary is the presence of polynuclear ova and polyovular follicles (Pl 1, figs 1-4). These multiple forms are found in the ovaries of animals killed during proestrus, estrus and also at certain times during pregnancy. Counts were made of the number of multiple forms per 1000 oocytes in each ovary. Such counts are necessarily only an indication of the relative occurrence of the multiple forms.

Polynuclear ova were not found until 3 days before the onset of heat, when they numbered 4% of the oocytes counted. The number increased during estrus to 12% and remained high until the 3rd day of the cycle. From this time onwards counts became increasingly difficult to make as the wave of degenerative changes affecting the ovary made the differentiation of a particular type of follicle uncertain. Polynuclear ova are mainly limited to the primordial stage of oocyte development, but some are found in follicles possessing two or three layers of granulosa cells. Vacuolation of the ooplasm and inequality of the nuclei are often found and are probably one of the first indications of impending degeneration (see Hartman, 1926). Binuclear oocytes are the most frequent type to be encountered, but as many as ten nuclei have been found in one oocyte. The volumes of these polynuclear ova (average diameter of 50 oocytes with 2 nuclei = 36μ , of 6 oocytes with 3 nuclei = 39μ , of 3 oocytes with 6 nuclei = 60μ) indicate that the condition is probably the result of the fusion of varying numbers of oocytes (see Hartman, 1926). Further support is provided for this theory by the presence of small spoke-like processes in the ooplasm of the polynuclear ova, which probably represent the remnants of the cell membranes of the original oocytes (Pl 1, fig 3).

Polyovular follicles were first found in ovaries obtained 4 days before the onset of estrus and numbered 5% of all oocytes. The number rose to 12% in ovaries obtained 1 day before the onset of estrus. On the 1st day of estrus 10% of the oocytes showed the polyovular condition, but the number fell to 4% on the 3rd day of the cycle. Hartman has classified polyovular follicles in three types. In Type I the ova are separated by intervening masses of granulosa cells, in Type II the ova are in contact by broad surfaces, and in Type III the ova are linearly arranged within the follicle. In the goat the polyovular follicles are most commonly of Type II (Pl 1, fig 2), examples of the other types being rarely found. Accessory oocytes are occasionally found enclosed in the periphery of a follicle, and one small oocyte was found in the ooplasm of a larger degenerating oocyte.

The signs of impending degeneration are seen earlier in the polyovular follicles and polynuclear ova than in normal oocytes in the same ovary. Only one in many thousands of multiple follicles observed had progressed beyond the primordial stage. This was a bimovular follicle that had reached the diameter of 550μ , and which contained two follicular cavities separated by a septum of granulosa cells. At the peripheral part of the septum theca interna cells were present as a small projection, a few cells thick, dividing the granulosa cells of the septum into two layers. One of the ova showed signs of degeneration.

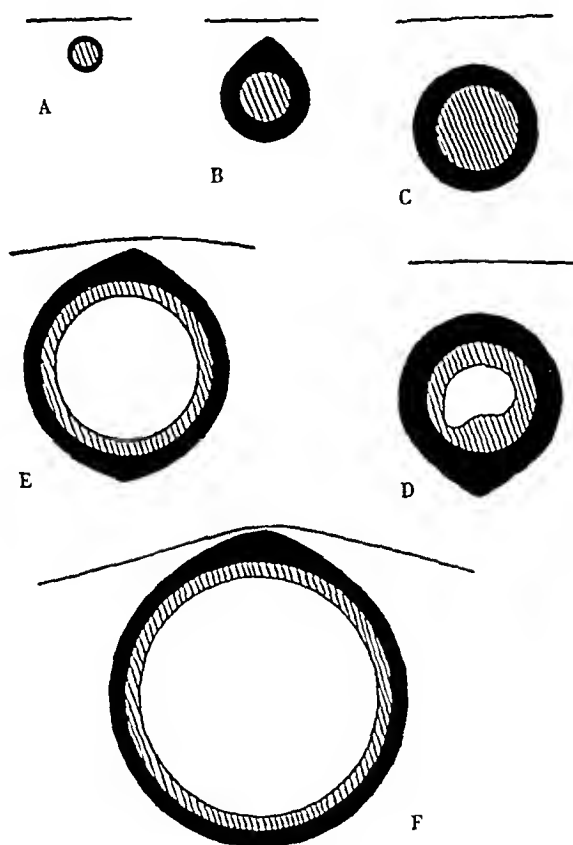
FOLLICULAR DEVELOPMENT

The ovarian follicles in the goat ovary change little in size during anestrus and metestrus. All ovaries obtained at these periods contain a few medium-sized follicles (2–5 mm) and a large number of small follicles (0.5–1.0 mm). Many of these follicles show signs of degeneration. There is considerable activity noticeable in the small and medium-sized follicles 5–6 days before the onset of heat. Both membrana granulosa and the theca interna cells show numerous mitotic figures and there is a considerable increase in the number of cells composing both layers (Pl. 2, fig. 10). The theca interna is often far thicker than the membrana granulosa in the developing medium-sized follicles, and in some follicles is at least twenty cells thick. The theca interna at this stage has the characteristics of a 'thecal gland', using the term as applied by Mossman (1937) to the great enlargement seen in the theca interna of *Geomys*. The thecal gland of the goat is apparently larger than that seen in the other domestic ungulates. The theca interna does increase in thickness at estrus in the sow (Corner, 1919), in the cow (Hammond, 1927), in the sheep (Warbritton, 1934) and in the mare (Harrison, 1946), but in most of these animals the theca interna seldom exceeds the thickness of the membrana granulosa.

The cells of the theca interna of the smallest follicles are flattened and arranged tangentially about the periphery of the developing follicle. The nuclei are fusiform, stain heavily, and the cytoplasm of the cells is scanty in amount and devoid of granules (Pl. 2, fig. 9). However, during proestrus, the cells increase rapidly in number and size, the nuclei are less heavily stained and are vesiculated. The cytoplasm increases in amount and many cells show increased granulation. The shape of the cell is now polygonal, and the nuclei are spheroidal or oval (Pl. 2, fig. 10). This activity of the theca interna is most marked nearest the ovarian surface, thus producing marked thecal cones such as Strassmann (1941) noticed in the small follicles of the cat, the rabbit and the mare.

The appearances of the thecal cone during different stages of follicular development suggest that the function of the cone is not as simple as Strassmann states. In the young follicles, under 300μ in diameter, a marked thecal cone can be seen at the side of the follicle nearest the surface of the ovary (Pl. 1, fig. 6, Text-fig. 1B). The cells in this cone are large and polyhedral, and there is evidence of much mitotic activity. The cells at the opposite pole of the follicle are fusiform and flattened tangentially against the membrana

propria. From the general appearance of the follicle it would seem that this proliferation of the cells of the theca interna pushes the follicle into the stroma of the ovary, thus causing the long recognized 'sinking in' or migration of the primordial follicle. After the follicle has migrated inwards the primary thecal cone is no longer visible and the majority of the follicles show an equal development of the theca interna all round the follicle (Text-fig 1C). However,



Text fig 1 Diagram to show the changes in shape of the theca interna in the maturing follicle. See text for explanation of the stages

follicles can be found, particularly where migration inwards is slowed up by the presence of old corpora lutea or neighbouring follicles, in which there is a distinct enlargement of the theca interna at the inner, or medullary pole (Pl 2, fig 7, Text-fig 1D)

The formation of the thecal cone of young follicles may be due to the better blood supply to the cortical pole of the follicle, for it has been shown that in the mammalian ovary the ovarian artery divides at the hilum into cortical and medullary branches (Andersen, 1926, and Bassett, 1943). Later in follicular

development two wreaths of capillaries appear, one in the theca externa, and the second in the theca interna and presumably the now evenly distributed blood supply is responsible for the equal development of the theca interna all round the follicle. In the same way the development of the internal thecal cone may be due to arrested migration of the follicle when its inner pole has just entered the area of the ovary supplied by the medullary branches of the ovarian artery, and before the two capillary wreaths have developed.

Harrison (unpublished) has shown that the administration of follicle stimulating hormones to rabbits, in which the immature follicles had been experimentally ruptured with the removal of the granulosa cells, resulted in a greater increase in the activity and thickness of the theca interna layer than occurred in the controls (see also Westman, 1934). This effect, and also that of the formation of a thecal cone (see Strassmann, 1941), may be due to the follicle stimulating hormone, or to the oestrogens secreted as a result of follicular stimulation, acting on the theca interna (see Bullough, 1946).

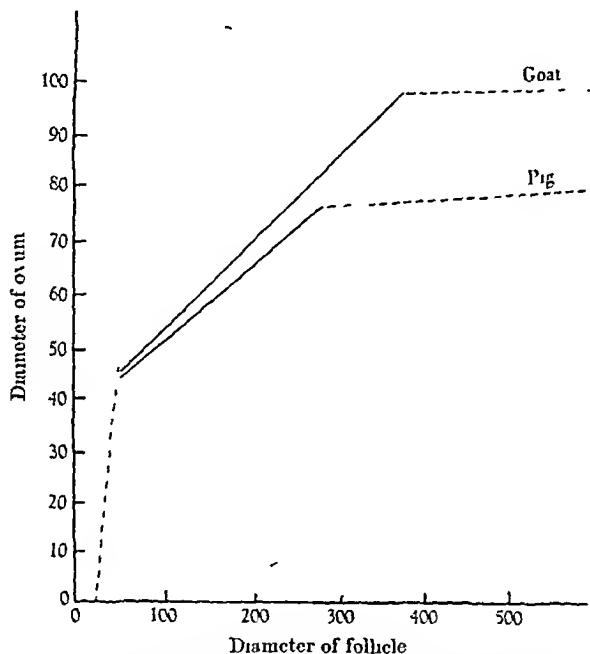
As the follicle develops further it pushes towards the ovarian surface, at first merely by its own relative increase in size, but later the outer cells of the theca interna re-enter the cortical zone of the ovary. Thereupon a second external thecal cone develops in the maturing follicle (Pl 2, fig 8, Text-fig 1E, F), and possibly takes part in the causation of ovulation, as described by Strassmann. In the goat ovary, as the follicle enlarges prior to ovulation, the marginal theca externa cells and those of the tunica albuginea are reduced in number and the tissue becomes oedematous at this point. The theca interna cells appear to invade the theca externa cells, but there does not appear to be any dissolution of the cells in the area, such as the postulated presence of a proteolytic enzyme in the follicular fluid by Schochet (1916) might suggest. At maturity the theca interna cells are well marked about the periphery of the rest of the follicle, but the layer is thinner than it was at the time of maximum development of the thecal gland. This thinning is presumably due to the sudden secondary enlargement of the follicle occurring at the end of proestrus. The alterations in the shape of the theca interna that may occur during the development of the ovarian follicle are illustrated in Text-fig 1.

FOLLICULAR GROWTH

It has been seen that at proestrus all types of follicles are influenced by some growth stimulus which causes them to increase rapidly in size. The precise histological effect of this is shown by the reaction of the theca interna and membrana granulosa already described. Therefore, in the ensuing description, the term 'actively growing follicle' will only be applied to those follicles which show these well-marked changes. Such actively growing follicles are only found during proestrus, estrus, and during a certain period in pregnancy. It appears that the primary oocytes develop as far as the small follicle stage (0.5–2.0 mm) during proestrus and estrus, and then remain quiescent probably until the next proestrus. If they have survived the wave of atresia affecting

the ovary during the end of estrus and in postestrus, they are presumably affected by the growth stimulus at the next proestrus and enlarge rapidly to a possible maximum diameter of 8–10 mm. The minimum time taken for the development of the follicle to maturity is therefore one diestrous cycle.

The growth of the ovum and follicle from the primordial stage until maturity can be divided into the usual two well-marked phases as described by Brambell (1928) and Parkes (1931). In the first phase the ovum and follicle grow concurrently, whilst in the second phase the follicle grows rapidly in size, whereas the relative increase in size of the ovum is slight. By measuring the diameters of a large number of actively growing follicles and treating the results statistically



Text fig. 2. Graph to show the growth of the ovum plotted against the growth of the follicle in the goat and the pig (Parkes, 1931). For the equations of the regression lines, see text.

in the manner described by Brambell (1928) the following formula for the regression line of the first phase is obtained

$$y = 32.52 + 0.156x,$$

where $x = 80-400$. Measurements were made only on material fixed in Bouin's fluid so that the results might be comparable to the work of Parkes (1931). The largest mean diameters were taken of developing ova and growing follicles, any follicle or oocyte which showed signs of atretic or degenerative changes was discarded. The goat oocyte, like that of the pig ovary (Parkes, 1931), reaches a diameter of 45μ before acquiring an entire epithelial covering. The diameter of the follicle when the antrum appears is $400-450\mu$ (pig $= 400\mu$),

and the diameter of the follicle is 400μ (pig = 300μ) when the growth of the oocyte reaches the end of the first phase

Only a few ova were found in the largest follicles that did not show signs of degeneration. Thus the formula for the regression line of the second phase, in which the ovum increases 50μ in size, whereas the follicle enlarges rapidly to 8 mm, could only be estimated from a relatively large number of measurements on medium-sized follicles. From all the available figures a formula of

$$y = 93.6 + 0.0058x,$$

where $x = 450-8000$, was obtained. Assuming that the follicle has a diameter of the order of 8 mm when it ruptures at ovulation, this regression line corresponds quite well with the diameter of the goat egg given by Hartman (1929) and Amoroso, Griffiths & Hamilton (1942) as an average figure of 145.3μ . Comparing these formulae with those of the pig (Text-fig. 2), given by Parkes (1931) as phase *a*, $y = 35.22 + 0.1382x$ and phase *b*, $y = 74.95 + 0.0049x$, it will be seen that the regression lines are similar to those of the goat. In the goat, however, there is a greater increase in the size of the oocyte during phase *a*, for the oocyte in the goat reaches a diameter of 95μ as opposed to that of 76μ in the pig. The ratio of the size of the follicle when the ovum is fully grown to the size of the mature follicle is 1:20, which can be compared with that of 1:26.6 for the pig (Parkes, 1931).

FOLLICULAR ATRESIA

Degeneration of the sex cells occurs continuously throughout the estrous cycle, but the process reaches a maximum in the goat ovary during the third and fourth days after the onset of estrus. In the ovaries of the animals killed at this time healthy primary oocytes are rarely to be found. Extensive degeneration of follicles of all sizes occurs later in the cycle than of the oocytes, but in some of the medium- and small-sized follicles early atretic changes can be detected during proestrus. Degeneration of the large follicles occurs later still, for it is often possible to detect large follicles that appear healthy by histological criteria in the ovaries of animals killed during estrus and early post-estrus. It would appear, therefore, that a wave of degeneration starts to affect the follicles at, or just before, the onset of estrus. The follicles appear to be affected in the order of their relative size, the largest follicles remaining healthy the longest time. Smaller numbers of follicles also show early atretic changes at other times of the cycle, but the causation of this limited atresia is probably not due to the factors causing the widespread destruction described above.

In the primordial follicles the first histological evidence of degeneration can be seen in the oocytes rather than in the follicular elements. The ooplasm swells, becomes vacuolated, and the nuclei undergo fragmentation. In many oocytes the cell membrane is destroyed and cells, which are probably granulosa cells, are found inside the degenerating oocyte. It is not yet clear whether these presumed granulosa cells are acting as phagocytes, or whether the oocyte

possesses phagocytic powers itself at this stage (Asami, 1920) The granulosa cells quickly degenerate and disappear, leaving a small hyaline plug surrounded by a nest of thecal cells to mark the site of the degenerated oocyte

In follicles that possess an antrum degenerative changes in the granulosa cells are an early feature of impending atresia Usually atretic changes in the granulosa cells appear after, or coincide with, early degenerative changes in the oocyte The oocyte is often collapsed or shrunk, and the cumulus oophorus is less marked Occasionally large atretic follicles are found in which the liquor is present as a fine-meshed coagulum, instead of the flocculent precipitate of coarse mesh of healthy follicles Such an alteration in the character of the follicular fluid was found by Kingsbury (1939) to be a constant sign in early atretic follicles of the cat, but in the goat, however, atretic follicles can be found which do not show this change The inner granulosa cells are loosely arranged and many of the cells are floating free in the follicular cavity At first the peripheral granulosa cells next to the basement membrane retain their columnar arrangement, but they are soon dispersed by a rapid ingrowth of theca interna cells The granulosa cells swell slightly as they degenerate, their nuclei then become pyknotic and fragmented, and the cells assume the appearance of leucocytes

The follicle collapses rapidly after the atretic changes have appeared in the granulosa cells Large numbers of spindle-shaped thecal cells, the majority derived from the theca interna, pass into the cavity of the collapsing follicle The thecal cells are enlarged, and contain numerous fat droplets In some cases a layer of amorphous material can be seen between the degenerating granulosa cells and the invading thecal cells, this layer has been called the 'glass membrane' (Shaw, 1926) It is difficult to decide the exact nature of this material, but it appears to be derived from the degenerating granulosa cells The latter cells eventually disappear entirely, leaving the old follicular cavity filled with a varying amount of the eosinophilic amorphous material The thecal cells continue to grow in towards the centre of the collapsed follicle, passing through the amorphous material in radiating strands of cells Mitotic figures are common in the thecal cells at this stage

At a later stage the entire follicular cavity is filled with interlacing thecal cells, though a small central plug of amorphous material, which may have become hyalinized, often remains for some time The thecal cells have now shrunk considerably and have the appearance of typical fibrocytes, often being surrounded by collagen Leucocytes are rarely seen in the atretic follicles at this late stage

In large follicles the granulosa cells degenerate in the manner described above The majority of the large follicles lose all their granulosa cells, which float off into the follicular fluid, and are destroyed There is seldom an invasion of the large atretic follicles by thecal cells, however, and the follicle becomes a cyst-like cavity with its smooth walls lined by the 'glass membrane' The theca interna cells are often swollen, with vacuolated cytoplasm, and their

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basal layer, which still retains its columnar structure, and the inner zone, which is loosely arranged in the antrum of the follicle. The granulosa cells soon show the usual signs of degeneration and rapidly disappear. The subsequent stages of atresia are the same as are found in the follicles of non-pregnant animals.

The changes that occur in the granulosa cells of atretic follicles of pregnant animals are similar to those that occur in lutealization. In the pregnant mare Colc & Hart (1930) have found marked lutealization of the follicles starting at the 40th day and reaching a maximum by the 110th day. At this time the follicles have become filled by fully lutealized granulosa cells, apparently resulting in the formation of numbers of accessory corpora lutea. Thus these changes of apparent lutealization, transient in the goat and other mammals and presumably merely showing what Martínez-Estève (1942) has called 'a flicker of a response to the luteinizing hormone', are marked and progressive in the mare.

In several follicles of the animals killed after the 45th day of pregnancy the thecal walls have been denuded of granulosa cells, and the appearance is now that of a cyst. In several of these cyst-like degenerating follicles of pregnancy a localized proliferation of theca interna cells is found. These thecal cells are large, their nuclei are vesicular, and mitotic figures are common. The proliferations of the theca interna cells form tufts growing into the follicular cavity, and as the follicle collapses these tufts nearly fill the cavity. Such proliferations of theca interna cells were only noted in degenerating follicles in which the granulosa cells had almost disappeared (see Culner, 1945, 1946 for changes simulating those of lutealization in the theca interna cells of human and baboon ovaries).

THE DEVELOPMENT OF THE CORPUS LUTEUM

The corpora lutea available for examination range from the time of ovulation to the 21st day in non-pregnant animals, and to the 60th day in pregnant animals. The approximate ages of the corpora lutea obtained from the 22 goats in which the onset of estrus had been determined biologically is given in Table 1. As has already been explained, it is assumed that ovulation occurs late in estrus, probably 30–36 hr. after the onset of full estrus, and the ages of the corpora lutea are given accordingly.

FIRST DAY AFTER OVULATION

The earliest ruptured follicles obtained, seven in number, are from goats 28, 40, 13, 26, and extend over the period ± 6 hr. to ± 12 hr. after ovulation. The freshly ruptured follicle measures $5.5 \times 4 \times 4$ mm. on the average. The collapsed follicles show varying degrees of plication of the walls, in some specimens there is barely any detectable folding of the mural epithelium, the collapsed follicle being elongated, with a slit-like cavity separating the walls. This type is always found when a ruptured follicle is close to the retrogressing corpus luteum of

the previous cycle. In the remainder there is a moderate degree of plication, never as marked as in the ovary of the mare, and hardly as marked as in the sheep or the sow. The stigma is generally oval, and was of no use in estimating the age of the corpus luteum. Herniation of the gland was found in a few of the early specimens.

In all the early ruptured follicles there is slight haemorrhage into the central cavity, the blood originating from ruptured thecal vessels. In places small channels are formed through the membrana granulosa through which blood can be seen percolating towards the central cavity. These channels appear to be associated with the future migration of endothelial cells from the thecal capillaries into the gland. The central cavity contains fibrin, in the form of fine strands, enclosing erythrocytes, leucocytes and some detached granulosa cells.

The granulosa cells in the earliest of the specimens are separated by slight spaces filled with fluid. The general appearance is of the 'compact' type of arrangement of the granulosa cells (Pl 3, fig 12), with only slight disassociation of the individual cells other than at the site of cleavage due to extravasation from the thecal vessels. In no specimen of the series was there an open 'lace-like' arrangement of the granulosa cells, as described in the dog (Evans & Colc, 1931), the fox (Pearson & Enders, 1943), or the mare (Harrison, 1946). The changes of lutealization appear relatively early in the goat, for in all the specimens the granulosa cells have increased in size to 15μ and the cytoplasm is evenly filled with granules. The granulosa cells are mostly spherical, with fluid filling the spaces between them, but at the tips of the folds elongated cells are streaming into the cavity of the collapsed follicle. The thickness of the mural epithelium is already two to three times that of the ripe follicle. Many of the nuclei, particularly those of the cells at the periphery of the epithelium have lost the dense compact arrangement of the chromatin. This has become broken into discrete masses or rods. Mitotic figures were not observed in the granulosa cells.

The theca interna cells are clearly visible, their position and local distribution depending on the degree of plication present. In the less folded type the cells are arranged fairly uniformly round the periphery of the granulosa cells, but with local aggregations, several layers thick, on the inner side of the ruptured follicle. The cells are spindle-shaped, with elongated nuclei, which do not stain always as heavily as the granulosa cells. The nuclei often show evidence of vesiculation. On the inner side of the ruptured follicle the theca interna cells are often radially arranged in the compact manner found in the ripe follicle prior to ovulation. In the collapsed follicles that show more plication the thecal cells are mainly distributed at the base of the central core of the folds (Pl 3, fig 12). They are arranged at the side of the central strands of theca externa cells and thecal vessels that are drawn into the centre of the core. These cells are plump, with a spherical nucleus, and their cytoplasm is evenly filled with numerous small vacuoles.

The thecal vessels are all engorged at this early stage particularly in the cores of the folds (Pl 3, fig 12) Endothelial cells can be seen migrating into the cores, and although it is difficult to be certain, in one or two of the early ruptured follicles spindle-shaped cells, quite unlike thecal cells, can be seen in the periphery of the granulosa layer These cells have definitely migrated through the membrana granulosa and in several places such cells can be seen disrupting the membrana propria Strands of an even amorphous material are laid down around these cells, and also in the wake of their migration This material stains well with Mallory's, Masson's and various silver stains, and is apparently reticulum There has been considerable discussion as to the nature of these cells and the material that they lay down at this early stage It has been suggested that they are theca interna cells which have reverted to fibroblasts, that they are endothelial cells behaving as the reticulo-endothelial cells which are found in the liver and suprarenal, or that they are typical theca externa or stromal cells (see Corner, 1919, 1920, 1921, 1945 for further discussion) The appearances in the goat support the view of Corner that these cells are endothelial cells that break away from the thecal capillaries and pass through the disrupting membrana propria to form the endothelial sinusoids of the gland, as well as the supporting reticular matrix of the fully developed corpus luteum

SECOND DAY AFTER OVULATION

Two specimens show the changes occurring from 24 to 48 hr after ovulation (Pl 3, fig 13) By this time the developing corpus luteum has almost filled the central cavity The boundary between the granulosa cells and the theca interna cells is now obscure, and the membrana is thin or has disappeared There are no longer any fluid-filled spaces between the individual cells The granulosa cells are larger, with an average diameter of 25μ and a nucleus of 9μ Granules are now evenly distributed in the cytoplasm of most of the granulosa cells, but a few cells at the periphery show some clearing of the granules and numbers of small spherical vacuoles have appeared at the periphery of the cell The nuclei of the granulosa cells are less dense and have all taken on the characteristic vesicular appearance With the increase in size of the granulosa cells the intercellular spaces have entirely disappeared and the typical compact arrangement of the gland is seen

The theca interna cells have altered little in position from the preceding stage, and are still arranged about the periphery of the granulosa layer, particularly at the bases of the folds The folds have thickened slightly by the passage into them of more theca externa cells The theca interna cells have now a relatively darker staining nucleus than those of the granulosa cells, and their cytoplasm is more vacuolated than in the previous stage Endothelial invasion is now well marked, and the cells seem to sprout out in chains from the ruptured thecal capillaries, both at the periphery of the gland, and from the cores of the folds Definite areas of early reticulum deposition can be seen

at the periphery of the gland. Invading thecal cells can rarely be found in these areas, whereas chains of endothelial cells are quite common. It therefore seems probable that the reticulum is laid down at this stage solely by the endothelial cells. Leucocytes are now found evenly distributed over the granulosa layer, as well as in the remnants of the central cavity. The developing corpus luteum now has an average measurement of 6-7 mm in diameter.

THIRD TO SEVENTH DAY AFTER OVULATION

Seven specimens are available to cover this period. During the 3rd-7th day the corpus luteum reaches a diameter of 9 mm. This corresponds to the time at which the blastocyst is implanted on the 7th day (Amoroso, Griffiths & Hamilton, 1942). The central cavity is completely obliterated during this period by the hypertrophy of the granulosa cells, which are now fully lutealized and have an average diameter of 30μ . The concentration of the granules is now markedly perinuclear and the periphery of the cytoplasm is filled with numerous vacuoles of different sizes (Pl 3, fig 14). The nuclei are large ($11-12\mu$), spherical and vesicular.

During this period the theca interna cells are difficult to trace. There are few mitotic figures to be seen in the theca interna cells during this period, and the relative scarcity of the cells is made more noticeable by the great hypertrophy of the granulosa cells. In the goat the theca interna cells can be easily distinguished again by the 5th day after ovulation. The cells are now evenly distributed throughout the entire gland and can be recognized by their small size (15μ in diameter). Their nuclei stain more densely than those of the granulosa cells, and their cytoplasm contains evenly distributed vacuoles, usually small and numerous, giving the cells a foamy appearance. Occasionally, however, there is only one large vacuole present.

By the 7th day a network of capillaries has become uniformly distributed within the entire gland, nearly every cell being surrounded by an endothelial coat. Associated with the increased vascularity of the gland thin-walled venous channels have appeared during these last few days, first at the periphery of the gland, then later radiating from the centre. Even in fixed specimens these channels are of a relatively large calibre. This is evidence of the formation and active functioning of an arterio-capillary-venous circulation which is rapidly replacing the 'early lymph-like drainage' (see Corner, 1945, and van der Horst & Gillman, 1946).

TWELFTH DAY AFTER OVULATION

Two specimens are available for examination of the appearances at this time. The corpus luteum has now reached its maximum diameter of 11 mm (Pl 3, fig 15). Most of the granulosa cells are large, with a diameter of $30-40\mu$. The cytoplasm of these cells is heavily vacuolated, but there are also cells which do not show vacuolation, having an almost homogeneous heavier staining

cytoplasm The vascularity of the gland has now reached its maximum, but there is little blood in the vessels, possibly due to the pressure exerted by the fully developed gland The theca interna cells are similar in appearance and distribution to the last stage

CORPUS LUTEUM OF NON-PREGNANT ANIMALS

Corner (1921) has shown that there is a sudden change in the corpus luteum of the sow at the 14th or 15th day after ovulation and that retrogressive changes then commence In the goat retrogressive changes also commence at this time, but they are not so abrupt as Corner describes in the sow During the period between the 12th day and the onset of the next estrus the luteal cells slowly degenerate It is naturally difficult to say at which day the luteal cells cease to produce progesterone Examination of the reproductive tracts of the goats used for this paper (unpublished data) indicates that there is a gradual decrease, starting at the 12th day, in the amount of multi-layered epithelium of the uterine lumen, which is being replaced by a uniform single-layered epithelium Lymphocytic infiltration, the relative numbers of goblet cells and the amount of mucous secretion also diminish gradually during the period extending from the 12th to the 19th day The changes occurring in the vagina, the lower uterine segment and the fused horns also suggest a gradual decline in the luteal action on the reproductive tract from the 12th day onwards From this evidence, and from that obtained from the histological appearance of the corpus luteum, it would appear that the corpus luteum, although some of its cells may still be functioning, ceases to have a full effect on the reproductive tract from the 12th day after ovulation

The five specimens of corpora lutea that were obtained during the period between the 12th and 20th day after ovulation all show retrogressive changes in the luteal cells The amount of cytoplasm is considerably reduced, the cells having an average diameter of 20μ by the 20th day Vacuolation at the periphery of the cytoplasm is still a marked characteristic during the 12th to the 17th day Cells are also seen in which the peripheral vacuoles appear to coalesce and the perinuclear cytoplasm shrinks round the nucleus (Pl 3, fig 16) Many cells can be seen in a corpus luteum of the 18th day after ovulation showing this contraction of the cytoplasm The latter now stains brightly with eosin and contains large numbers of minute vacuoles Such an appearance of the cytoplasm is reminiscent of the 'mulberry' cells which Corner (1945) described in the degenerating luteal cells of the rhesus monkey Occasionally some luteal cells of the goat corpus luteum also show the 'giant' vacuoles that Corner described Associated with this perinuclear shrinkage of the cytoplasm degenerative changes are seen in the nucleus (Pl 4, fig 18) The chromatic elements become massed round the nuclear membrane in the form of spheres These spheres rapidly coalesce to form the typical pyknotic appearance, and this is followed by fragmentation and disappearance of the nucleus

The general appearances of the degenerating gland rapidly become those of

a mass of compressed and occluded capillaries, surrounded by masses of stromal fibrous tissue. The arterioles and veins have obtained adventitious sheaths of fibrous tissue, and in the meshes of the latter are numbers of degenerating luteal cells. The theca interna cells can no longer be discerned by the simpler staining methods. It is difficult to decide whether they have disappeared at an earlier stage, or whether they have reverted to fibroblasts. There is no 'interstitial gland' in the goat ovary, similar to that in the rabbit ovary, thus there is no question of the theca interna cells persisting as the cells of such a structure. It appears that the theca interna cells are probably destroyed in the general degeneration that overtakes all the parenchymatous elements.

Numerous degenerating corpora lutea are available from the series and all show a progressive shrinkage with age. Corner (1945) has indicated a statistical method for the determination of the shrinkage in volume of the corpus luteum, but the results in the goat are subject to too many sources of error for the method to be more reliable than a simple statement of the mean result of the three spatial diameters. From a mean diameter of 11 mm. at the 12th day the corpus luteum shrinks to 8 mm. on the 20th day. There is then a rapid shrinkage, following the next estrus, to 6.5 mm. on the 23rd day. By the 40th day there has been a steady shrinkage to 4 mm. and by the time the old corpus luteum has been subjected to two oestrous cycles it is 2.5 mm. in diameter. Thereafter the corpus luteum is represented by whorls of fibrous tissue surrounding the degenerated remnants of the blood vessels, the original site of the gland being a dense mass of fibrous tissue covering an area of about 1 mm. in diameter. A few degenerated luteal cells can be seen for 3-6 weeks, interspersed in the fibrous tissue, and showing numbers of large vacuoles. In most cases the nuclei have degenerated, and there is no evidence of any parenchymatous cell surviving the degenerative changes.

THE CORPUS LUTEUM OF PREGNANCY

The ages of the corpora lutea which were obtained from the animals that were mated can be seen in Table 2. The details of the stage of cleavage of the ovum recovered from the first five animals in the series can be found in the paper of Amoroso, Griffiths & Hamilton (1942). The appearances of the corpus luteum in these first five animals are similar to those given for the same stages in non-pregnant animals. There appears to be no way of differentiating the corpus luteum of pregnancy from that of non-pregnant animals during the first 7 days of development of the corpus luteum.

There is unfortunately a gap in the series of corpora lutea of pregnant animals from the 7th to the 20th day after mating, therefore no description of the corpus luteum at the time of implantation of the blastocyst can be given.

The appearances of the corpus luteum on the 20th day of pregnancy are similar in general to those already described for the 12th day after ovulation in non-pregnant animals. The diameter of the corpus luteum is the same as that of the 12th day (11 mm.), but the luteal cells have shrunk slightly to an

average diameter of 30μ . The cytoplasm of the luteal cells contains only a few vacuoles, and this may be the cause of the smaller size. The theca interna cells can still be seen among the luteal cells and their cytoplasm is still markedly vacuolated. The capillary network is well marked, and the entire gland is hyperaemic (Pl 4, fig 19).

From the 35th to the 45th day of pregnancy there is a gradual shrinkage in the size of the luteal cells to an average diameter of 25μ . During this period three types of luteal cell can be found in the corpus luteum. In the first type, which occurs most frequently, there is considerable peripheral vacuolation of the cytoplasm. The second type, however, shows little vacuolation, and the cells of this type are smaller than the other types, their cytoplasm is more darkly staining and is heavily granular. The third type of cell is slightly larger, and can be distinguished by the presence of one or more large vacuoles in the cytoplasm (Pl 4, fig 20).

The shrinkage that has occurred in the majority of the luteal cells produces what Corner (1945) has called a 'decompression' of the gland. The result is an enlargement of the tissue spaces and an increased amount of connective tissue. A thickening in the walls of the larger vessels occurs at the same time.

At the 45th day the corpus luteum has again changed in appearance. The luteal cells have all enlarged to an average diameter of 30μ and there are no longer large vacuoles in any of the cells. Their cytoplasm has returned to its appearance on the 20th day, in which a homogeneous perinuclear area containing granules and some slight peripheral vacuolation were present. The increase in size of the luteal cells has obliterated the tissue spaces that temporarily appeared during the period from the 35th to the 45th day.

The general appearances of the corpus luteum at the 55th day (Pl 4, fig 21) are similar to those described for the 45th day. Some of the cells near the centre of the gland, however, have shrunk considerably. The cytoplasm is more heavily staining in these central cells, and no vacuoles are present in the cytoplasm. By the 60th day (Pl 4, fig 22) the majority of the luteal cells have undergone this contraction of their cytoplasm. The nuclei have also shrunk, and all the cells stain darker than those of the previous stage. The connective tissue appears to be increasing in amount, and the luteal cells are compressed by it into irregular shapes, and the capillaries in the centre of the gland no longer contain erythrocytes.

LUTEOLIPIN

Rossmann (1942) states that he was unable to find luteolipin in a series of corpora lutea of varying ages obtained from swine, sheep and cows. All the corpora lutea obtained from the series of goat ovaries under discussion were examined for luteolipin. In none was any substance found that fulfilled Rossmann's staining criteria for luteolipin. Treatment of the corpora lutea in varying stages of retrogression with Sudan III, after fixation in Zenker-formol, however, showed the presence of numerous spherical droplets in the cytoplasm.

of the degenerating luteal cells (Pl 4, fig 17). These cells could be followed through their retrogressive changes to the 6th week. At this stage a few cells remain, interspersed among masses of fibrous tissue and degenerated blood vessels. In haematoxylin and eosin preparations these cells can be recognized as 'ghost' cells, for they seldom possess healthy nuclei and are clearly degenerating. The material staining with Sudan III is apparently not luteolipin, but some higher form of lipid that resists alcohol extraction. Presumably the cells remain for so long due to the thick fibrous scar preventing their absorption.

DISCUSSION

Polynuclear ova and polyovular follicles (see Hartman, 1926, for discussion and references to literature) have seldom been reported in the ovaries of ungulates. Hellin (1895) believed that relative under-development of the connective tissue of the ovary leads to fusion of follicles and subsequent sterility. The presence of multiple follicles in the goat ovary at least provides evidence against this theory. The ovary of the goat contains as much, and possibly more connective tissue than the ovaries of the pig and the sheep. But even to accept the classical explanation that multiple follicles are an accident of development due to variable proportions of germ cells, epithelium and stroma in different animals is not entirely satisfactory. Hartman has suggested that the related phenomena of large numbers of primordial ova, polyovular follicles and polynuclear ova may be an expression of the same growth stimuli which begin in the embryo and continue during the reproductive cycle. In this connexion the suggestion of Baesich (1946) that the phenomenon is related to the effect of hormonal stimuli, and the work of Bullough (1946), appear relevant. The latter finds that in the mouse ovary increased mitotic activity in the germinal epithelium, with associated formation of new oogonia, is dependent on the local concentration of estrogens. Such mitotic activity in the epithelium is markedly increased in the neighbourhood of large maturing follicles containing estrogen. The formation of multiple follicles is found in the goat ovary when the estrogen production in the ovary is high and is suggestive of the action of this hormone. Baesich (1946) suggests that the relative frequency of multiple follicles in human new-born and infant ovaries is due to the withdrawal of gonadotrophic hormonal influences, but it must also be pointed out that in pregnant women the quantity of estrogen excreted in the urine increases throughout gestation until parturition (Newton, 1939). This estrogen is almost certainly produced by the placenta (Corner, 1938), but its action on the foetal ovary is not impossible.

Hartman has advanced the hypothesis that multiple follicle formation represents an atretic phenomenon, but it would appear more likely to be the result of an excessive stimulus, possibly estrogen, leading to inevitable atresia. Hartman states that the polyovular condition is independent of the polynuclear condition and that the former does not arise from the latter. In the series of goat ovaries examined it has been seen that during prooestrus there were

relatively more polyovular follicles than polynuclear ova, whereas during estrus there were relatively fewer polyovular follicles. Thus it is possible that many of the polynuclear ova may represent a stage in the degeneration of polyovular follicles. Indeed in many cases the remnants of what appear to be cell membranes can be seen in the polynuclear ova obtained during and after estrus.

Follicular growth in the goat ovary is in general similar to that described in the sheep by Grant (1934) and in the sow by Corner (1921), and the two phases of growth as described by Parkes (1931) in the sow have also been demonstrated in the goat. Until recently the two phases of follicular growth have been discussed from the point of view of the nutritional requirements of the growing ovum. Wimsatt (1944), in his paper on the follicular growth in the bat *Myotis lucifugus*, suggests that the growth potential of the follicular epithelium is inhibited by the necessity of furnishing nutriment to the developing ovum. The work of Bullough (1946), however, may throw new light on the factors responsible for the two phases. He has shown that the mitotic activity of the follicular epithelium in the mouse ovary increases when the antrum develops and the follicular cells are bathed by estrogen containing follicular fluid. The antrum develops in the follicles of the goat and the sow just after the oocyte has reached the end of the first phases of growth. Therefore it seems likely, assuming that Bullough's researches on the mouse ovary represent the state of affairs in the mammalian ovary, that during the first phase, when no antrum has appeared, the follicle does not increase greatly in size because there is a low concentration of local estrogens. When the follicular antrum appears, however, and the mural cells come into direct contact with the estrogen in the follicular fluid the second phase of marked follicular growth then commences. It is significant that the oocyte in many mammals goes through its first maturation division at a time when the antrum is appearing and the concentration of estrogen may be assumed to be increasing in the follicular fluid.

In general, the changes that occur during the development of the corpus luteum in the goat are similar to those occurring in the sheep and the sow (Marshall 1904, Grant, 1934, Corner, 1915, 1919). It appears that in the goat ovary, as in the ovaries of all the ungulates so far studied, the theca interna cells do not necessarily revert to fibroblasts, as has been asserted in the case of some mammals (Dawson, 1941). It also seems that the reticulum in the corpus luteum is laid down by the endothelial cells, and not by the theca interna cells as Solomons & Gatenby (1924) suggest. The fate of the theca interna cells will probably not be finally settled until a selective staining method for these cells is developed. Corner (1944) has made a preliminary investigation of the distribution of the theca interna cells in the corpus luteum of the sow using Gomori's method (1941) for the demonstration of alkaline phosphatase. He finds the theca interna cells, laden with phosphatase interspersed among the granulosa cells at the 18th day of pregnancy. His findings

with this technique completely corroborate his earlier description (1919) of the developing corpus luteum

The theca interna cells appear to degenerate eventually, and in none of the ungulate ovaries do the theca interna cells give rise to an interstitial gland. It might therefore appear that the statement of Ancel & Boun (1909) that animals which ovulate spontaneously do not possess a true interstitial gland, and that its place is taken by a periodic corpus luteum, receives some support from the state of affairs in the ungulate ovary. Pearson (1944) has shown that the converse of this statement is not necessarily true.

The stages of development in the corpus luteum of the goat can be compared with those recently described in other mammals. In *Elephantulus* three main periods of evolution of the corpus luteum of pregnancy have been described by van der Horst & Gillman (1946). In the first, which extends from the time of ovulation until the embryo is 10 mm long, there is a steady growth in the corpus luteum and it is indistinguishable from that of the menstrual cycle. The second period terminates when the embryo is 20 mm long, and during this period a large fat vacuole appears and disappears in each cell. During the rest of pregnancy there is an initial active growth of the corpus luteum succeeded by gradual degeneration. In the goat there appears to be an equivalent first stage of hypertrophy extending from ovulation until about the 35th day. Following this there is a similar period of readjustment or transition lasting for at least 10 days. During this phase numbers of the luteal cells show large vacuoles in their cytoplasm. Then follows a period during which the luteal cells at first hypertrophy once more, and then gradually appear to retrogress.

In *Elephantulus* there is marked follicular stimulation during the first phase of development in the corpus luteum. This occurs at a time when the chamber epithelium of the uterus is being eroded and when there is rapid differentiation of the decidual cells. In the goat, also, there is a definite precocious ripening of the follicles during the first 35 days of pregnancy. Giant (1934) noted an increase in the size of the corpus luteum of the sheep at about the 14th day, which is at the time when the destruction of uterine epithelium is commencing (Assheton, 1906). It therefore seems credible that the follicular stimulation that occurs during the early part of pregnancy is due, directly or indirectly, to the production of chemical or hormonal substances in the developing trophoblast (see Wislocki & Streeter, 1938). In the goat the increased activity in the follicles is associated with the production of new oocytes and also multiple oogonia. The theca interna of the precociously ripening follicles is well developed in the goat, and thus if the present view that the theca interna is responsible for the production of estrogen is correct (see Mossman, 1937, Corner, 1938), then the follicular growth and ovogenesis that occur at this time may well be caused by the increased amounts of estrogen. In this connexion the observations of Asdell (1946) and Cole & Hart (1942) that estrogen is first detected in the urine of pregnant mares from the 30th to 40th day of pregnancy appears

relevant Although the estrogen in the urine of pregnant animals is almost certainly produced by the placenta (see Corner, 1938, for review) the possibility that estrogen is produced in the ovary during the end of the first phase of hypertrophy of the corpus luteum has now to be considered

It has been shown that estrogens prolong the life of the corpus luteum (see Robson, 1937 and Allen, Danforth & Doisy, 1939) and thus the activity of the corpus luteum during the first phase may well be prolonged by the presence of estrogens As has been suggested above this estrogen is possibly produced in the ovary at first, and then later from the placenta

During the period of readjustment in the corpus luteum of the goat, which extends from the 35th to the 45th day at least, many of the luteal cells show a large vacuole, which later disappears A transition period similar to this is also present in the corpus luteum of the sow about the 40th day (Corner, 1915) It has been shown that there are three types of cell present among the luteal cells in the corpus luteum of the goat at this stage and these types probably indicate varying degrees in activity on the part of the luteal cells This period of readjustment has been associated by van der Horst and Gillman with the time at which the function and activity of the placenta is being established It is significant that in the ungulates generally it is from the 30th day onwards into the second month of pregnancy that the foetal membranes become fairly firmly attached to the cotyledons by finger-like processes that eat their way into the tissues of the cotyledon (Hammond, 1927) In the sheep Assheton (1906) states that by the 35th day there is a layer of trophoblast cells present everywhere in the crypts as a thin and irregular lining Examination of the foetal membranes of the specimens used for this paper indicate that a similar state of affairs exists in the goat

During the second stage of readjustment the follicles that had precociously ripened in the first phase are starting to become atretic and even cystic This may be due, as van der Horst and Gillman have suggested in the case of *Elephantulus*, to the action of progesterone Gillman (1942) and Gilbert & Gillman (quoted in van der Horst and Gillman, 1946) have shown that a single administration of progesterone in the baboon causes cystic follicles to appear, whilst prolonged administration of the hormone causes follicular atresia In the goat it is mainly the follicles in which an antrum has developed that take part in the precocious ripening during the first phase, possibly because the mitogenic effects of the estrogen then present surpass the atretic effects of progesterone

Estrogens also cause an increased secretion of the lutealizing hormone from the pituitary (Fevold, Hisaw & Greep, 1936) Thus the increasing amount of estrogen, presumably now produced by the placenta, may explain the changes that occur in the corpus luteum of the goat, the sow, and *Elephantulus* during the early part of the third phase of the development of the corpus luteum

SUMMARY

1 An account is given of ovogenesis, follicular development and growth, follicular atresia, and the development of the corpus luteum in the ovaries of 23 non-pregnant and 18 pregnant goats

2 Polyovular follicles and polynuclear ova are commonly found in ovaries of animals killed in proestrus and in estrus and also during a certain period of early pregnancy. Darkly staining bodies are found in the zona pellucida of precociously developed oocytes in ovaries obtained during late estrus. It is suggested that these bodies are the nuclei of granulosa cells incorporated in the zona pellucida

3 A marked thecal gland develops during proestrus. Localized proliferation of the theca interna gives rise to a thecal cone. Three types of thecal cone are distinguished during the development of the follicle

4 The usual two phases of follicular growth are found, the equations of the regression lines being phase *a*, $y = 32.52 + 0.156x$, phase *b*, $y = 93.6 + 0.0058x$

5 Atresia is maximal in the medium-sized follicles a few days after the onset of estrus. A precocious development of follicles occurs during the 20th to the 42nd day of pregnancy, which is followed by widespread atretic changes

6 The luteal cells are derived from the granulosa cells, and the theca interna cells are incorporated among the luteal cells. The theca interna cells do not give rise to reticulum, the latter apparently being derived from the endothelial cells

7 The corpus luteum in non-pregnant animals reaches its maximum diameter by the 12th day after ovulation. Many of the luteal cells show peripheral vacuolation at this time. The theca interna cells are difficult to trace after the 15th day and it appears that they degenerate after this time

8 The changes occurring in the retrogressing corpus luteum are described. The presence of luteolipin could not be demonstrated, but staining with Sudan III showed the presence of droplets of higher lipids in degenerating luteal cells for 6 weeks after ovulation

9 Four phases of development of the corpus luteum of early pregnancy are found. The first phase is one of steady growth and lasts for about 35 days, the second phase lasts at least 10 days and is marked by the appearance of a large vacuole in the cytoplasm of many of the luteal cells. During the third phase the vacuoles disappear, and in the fourth phase, commencing at the 60th day, many of the luteal cells have shrunk and are more darkly staining

10 The stages of development of the corpus luteum in the goat are compared with those described for other ungulates and are also discussed in the light of recent work on the corpus luteum of other mammals. It is suggested that the phases of development of the corpus luteum in the goat are related to the development and activity of the placenta

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EXPLANATION OF PLATES

PLATE 1

- Fig 1 Section through the cortex of an ovary from a goat killed 2 days before the onset of estrus Two binuclear oocytes are shown $\times 180$
- Fig 2 Section through the cortex of an ovary from a goat killed on the 3rd day after the onset of estrus Examples of polyovular follicles and polynuclear ova are shown, evidence of early degeneration is present $\times 250$
- Fig 3 Section through a polynuclear oocyte from an ovary of a goat killed in estrus Remnants of what are probably degenerating cell membranes can be seen in the oocyte $\times 500$
- Fig 4 Section through a polynuclear oocyte from an ovary of a goat killed on the 2nd day after the onset of estrus The oocyte contained ten nuclei altogether $\times 400$
- Fig 5 Section through a precociously enlarged oocyte from the ovary of a goat killed in estrus Elongated darkly staining bodies can be seen in the zona pellucida $\times 550$
- Fig 6 Section through a developing follicle in an ovary from a goat killed during proestrus The section has passed obliquely through the follicle and shows a well developed primary external thecal cone The oocyte was present in the remaining sections $\times 150$

PLATE 2

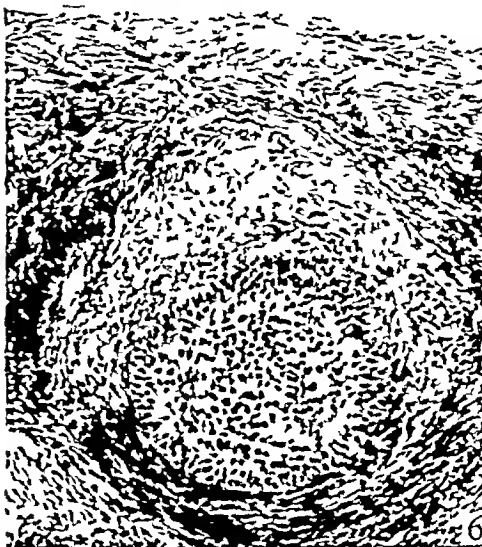
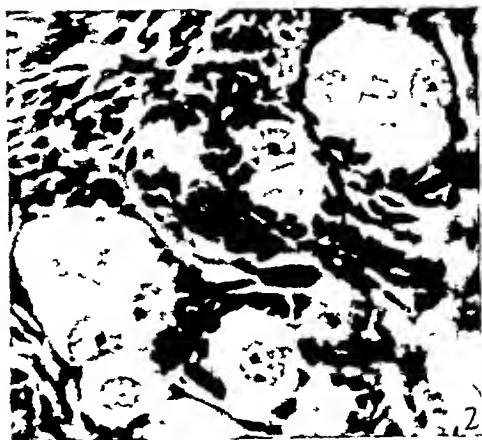
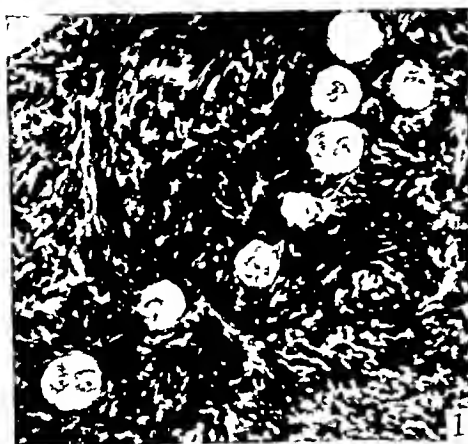
- Fig 7 Section through a developing follicle in an ovary from a goat killed in proestrus An internal thecal cone is shown $\times 70$
- Fig 8 Section through a developing follicle in an ovary from a goat killed in proestrus A developing secondary external thecal cone is shown $\times 70$
- Fig 9 Section through the wall of a maturing follicle on the 5th day before the onset of estrus The theca interna cells fill the area between the two white marks on the photograph $\times 600$
- Fig 10 Section through the wall of a maturing follicle on the 1st day before the onset of estrus The theca interna cells fill the area between the two white marks on the photograph $\times 600$
- Fig 11 Section through the wall of a precociously maturing follicle on the 33rd day of pregnancy The theca interna cells fill the area between the two white marks on the photograph $\times 600$

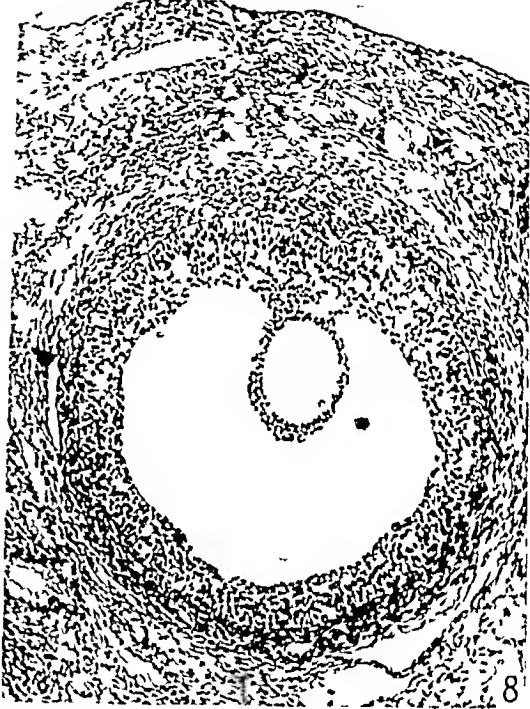
PLATE 3

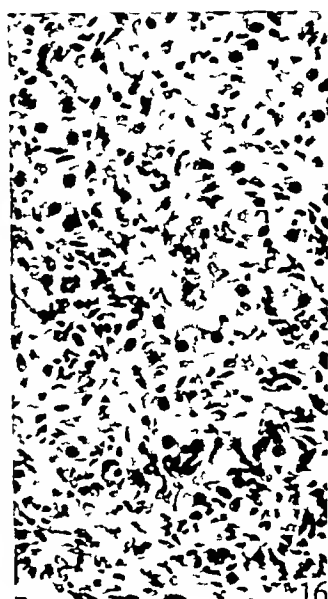
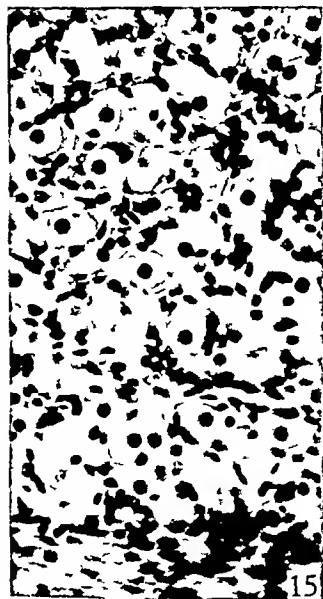
- Fig 12 Section through the developing corpus luteum The goat was killed 12-24 hr after ovulation $\times 70$
- Fig 13 Section through the developing corpus luteum The goat was killed 36-48 hr after ovulation $\times 70$
- Fig 14 Section through the developing corpus luteum The goat was killed 7 days after ovulation $\times 70$
- Fig 15 Section through the fully developed corpus luteum The goat was killed 12 days after ovulation $\times 90$
- Fig 16 Section through the degenerating corpus luteum The goat was killed 20 days after ovulation $\times 70$

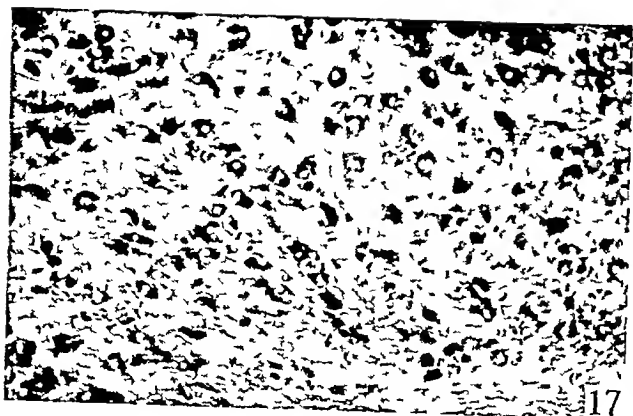
PLATE 4

- Fig 17 Section through a degenerating corpus luteum stained with Sudan III The corpus luteum is at least 24 days old $\times 70$
- Fig 18 Section through a degenerating corpus luteum The method of degeneration of the luteal cells is shown $\times 650$
- Fig 19 Section through the corpus luteum of pregnancy The animal had been pregnant for 33 days $\times 90$
- Fig 20 Section through the corpus luteum of pregnancy The animal had been pregnant for 46 days Large vacuoles can be seen in the luteal cells $\times 300$
- Fig 21 Section through the corpus luteum of pregnancy The animal had been pregnant for 55 days $\times 70$
- Fig 22 Section through the corpus luteum of pregnancy The animal had been pregnant for 60 days $\times 70$





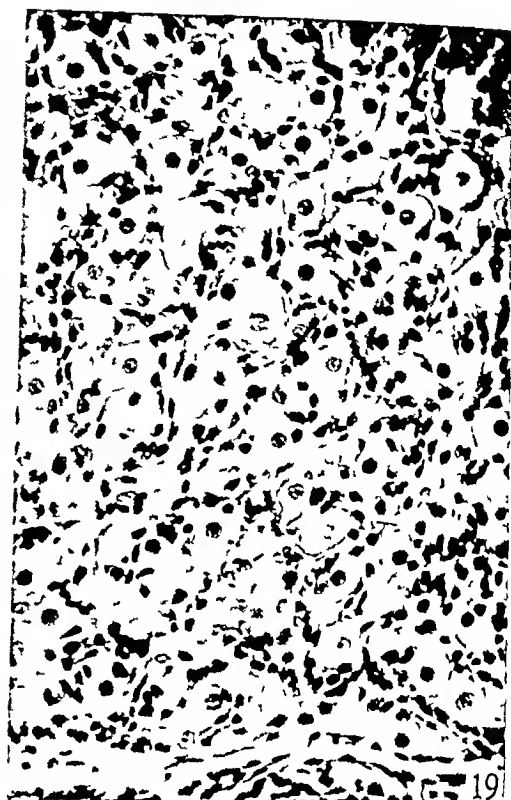




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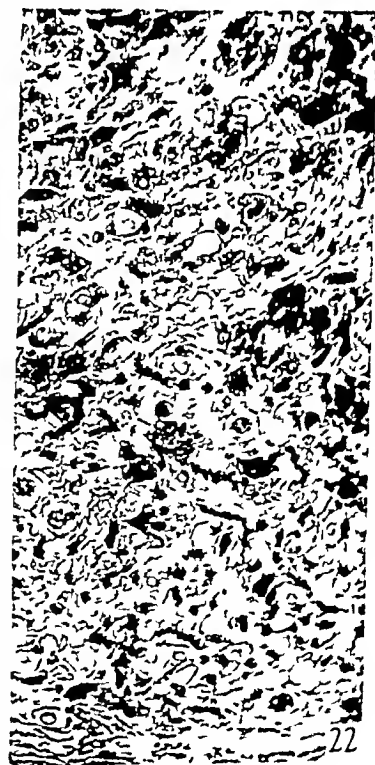
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HISTOPHYSIOLOGY OF THE LIMB-BUD OF THE FOWL DURING ITS EARLY DEVELOPMENT*

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INTRODUCTION

Although the morphogenesis of the fowl limb-bud has been studied fairly exhaustively (Fell 1925, Fell & Robison 1929, Fell & Canti 1934) little is known of the histochemical and physiological changes that occur in the tissues during differentiation. Observations on the chemistry of the developing limb-bud were made by Fell & Robison (1929), who investigated the alkaline phosphatase content before differentiation and after ossifying cartilage had developed. Phosphatase was not found in the undifferentiated limb-bud, but occurred in the ossifying rudiment, the amount of phosphatase being correlated with the amount of hypertrophic cartilage and bone which had been formed. Barnett & Bourne (1942) studied the distribution of ascorbic acid in tissues of the developing chick.

In the fowl the morphological developmental potencies of the various regions of the limb-bud are already determined before histological differentiation has begun. It therefore seemed interesting to discover whether any histochemical differences could be detected between areas of different developmental potencies before their characteristic histological differences were distinguishable.

The present investigation comprises a histochemical and cytological study of the limb-bud during its first $5\frac{1}{2}$ days of development. To define the developmental potencies of various regions experimentally, fragments of the limb-buds were excised and allowed to develop *in vitro*. The results recorded below show that by suitable methods certain histochemical and cytological differences can be demonstrated between areas of different developmental potencies, before any general cytological differentiation is visible. Some of the histochemical and cytological changes which the mesoderm undergoes in the course of differentiation are also described.

MATERIAL AND METHODS

The limb-buds of chick embryos ranging in age from 3 to $5\frac{1}{2}$ days were used.

(a) *General histological methods*

For general histological study the limb-buds were fixed in Bouin's fluid and dehydrated with the ordinary alcohol series or with dioxan (di-ethylene-dioxide). Sections were cut at 6μ and stained with either Delafield's haematoxylin and eosin or with Heidenhain's iron haematoxylin.

* This work was done in 1939 but, owing to the war and the author's unexpected departure for the Netherlands East Indies, publication of this paper has been delayed.

(b) Cytological methods

For more precise microscopical study the tissue was fixed in either Carnoy's fluid, or in Allen's or in Dubosc-Brasil's modifications of Bouin's fluid. The material was embedded in paraffin or celloidin-paraffin and the sections ($5-10\mu$) were stained with Heidenhain's iron haematoxylin, toluidin blue or Delafield's haematoxylin and eosin.

To demonstrate mitochondria the limb-buds were fixed in Champy's or Flemming's solution (without acetic acid) and stained with iron haematoxylin. The mitochondria were also examined in fresh tissues. Longitudinal and transverse sections of the limb-bud were cut by hand at an approximate thickness of $50-100\mu$ and stained supra-vitally with Janus green B.

To show the Golgi substance the limb-buds were fixed in Champy's solution and then, after rigorous rinsing in tap-water, immersed in 1 % osmic acid for 9 days at 37°C . The sections ($3-5\mu$) were examined unstained.

The effect of vital staining with toluidin blue and neutral red was studied by immersing longitudinal and transverse sections of fresh limb-buds like those prepared for staining with Janus green B, in solutions containing 1:10,000–1:100,000 of the stain. Most of the sections were stained at room temperature, but some at 37°C . The lower temperature was preferable as the reaction proceeds more slowly and could be more easily followed. Some sections were observed continuously under high magnification from the beginning of the experiment so that the successive stages of the reaction could be watched.

(c) Histochemical methods

Fat. Transverse and longitudinal sections of limb-buds cut by hand were immersed in a solution of Sudan black or red in 70 % alcohol. Similar sections were placed in 2 % osmic acid and kept under continuous microscopic observation for 2 hr.

Limb-buds were also fixed in Champy's or Flemming's solution without acetic acid, and transferred to an alcoholic solution of sodium sulphide (one small crystal in 3 c.c. of 70 % alcohol) for 24 hr. The buds were treated with dioxan and embedded in paraffin wax.

Glycogen. Material was fixed in Carnoy's, Champy's or Allen's modification of Bouin's fluid and dehydrated in dioxan. It was then infiltrated with celloidin by a modification of Apathy's method recommended by Jacobson. The oil mixture consisted of 4 parts chloroform, 2 parts oleum origanum, 4 parts cedar-wood oil, 1 part phenol crystals. The limb-buds were embedded in paraffin wax and sections were stained with Best's carmine, Bauer's method and with Langhans' solution of iodine.

Ascorbic acid. The limb-buds were quickly rinsed in distilled water and immersed for 30 min., in the dark, in an acidified solution of silver nitrate (0.5 c.c. acetic acid in 10 c.c. 10 % silver nitrate solution). They were then washed, in the dark, for 1 hr. in repeated changes of distilled water and

embedded (again in the dark) in paraffin. The sections were treated with a saturated solution of hyposulphite or alternatively the entire bud was immersed in the same solution for 1-2 hr before embedding.

To obtain a better fixation than the above technique afforded, a new method was elaborated: the limb-buds were fixed in the dark for 30 min in Carnoy's solution, to which 10 % silver nitrate was added. Nucleus and cytoplasm were generally better preserved than in the preparations made by the previous technique and the ascorbic acid showed the same distribution.

(d) Tissue culture

A fragment of the limb-bud was excised and explanted by the hanging-drop method in a mixture of plasma and embryo-extract. The rest of the bud was fixed and sectioned so that the precise limits of the part removed could be determined.

The embryo extract was made with Pannett and Compton's saline. Most of the experiments were carried out with saline containing 1 % glucose (as suggested by Fell), which seemed to improve growth and differentiation. The explants were transferred to fresh media every 4 days.

OBSERVATIONS

The general histological development of the limb-bud during the first 5½ days' incubation

The limb-bud of the 3-day embryo shows no morphological differentiation and consists of mesenchyme with rather wide intercellular spaces and an irregular capillary network. The mesoderm at this stage appears syncytial after most fixatives, so that only dividing cells show a cell boundary, as previously stated by Fell (1925); this appearance is an artefact and in material fixed in Champy's fluid definite cell outlines are readily distinguishable (Fig. 1).

Differentiation begins in the proximal part of the bud where the mesenchyme condenses to form procartilage. The cells in the interior of this compact mass gradually become arranged in layers and soon differentiate into typical young cartilage cells. In the oldest stage examined (5½ days) cartilage was well developed and ossification had often begun. For further details of the histogenesis of cartilage and bone the reader is referred to the investigations of Fell (1925).

The differentiation of muscle is first distinguishable after 4½ days' incubation. In the proximal part of the bud some of the cells surrounding the stretched rudiment lengthen and grow out along the femur to form bundles of myoblasts. Fibrils appear in the cytoplasm and in the 5½-day rudiment are slightly but definitely striated.

The muscles of the tibial region differentiate later than those surrounding the femur and their site in the mesoderm is less clearly defined. In the metatarsal region the muscles begin to appear at about the 5th day of incubation. The histological picture suggests that the myogenic cells grow in a proximal direction from the end of the foot rudiment.

*Cytological and histochemical changes in the limb-bud**(a) The undifferentiated limb-bud (3 days)*

Although no morphological differentiation is visible at the 3rd day, regional differences of a histochemical nature can be demonstrated by appropriate methods

The cytoplasmic inclusions (mitochondria, Golgi complex, cell granules, fat) present the same appearance throughout the mesoderm. In material supravitaly stained with Janus green B, the mitochondria are seen as long, slender threads with occasionally some short, stout rods. Sometimes granular swellings can be observed in the filaments. The same picture can be found in well-fixed preparations.

The Golgi complex, which lies at one side of the nucleus, consists of a loose network (Fig. 1). Fat globules can be seen both in living cells and after post-vital staining with 2% osmic acid or with Sudan black or red. They occur throughout the cell but tend to congregate round the Golgi zone. The fat globules are difficult to fix, even when the limb-bud was immersed for 24 hr in 2% osmic acid at 37° C; they could rarely be stained unless the tissue was treated with sodium sulphide.

Vital staining with basic dyes shows marked differences in various cell-layers. After 15 min. treatment with toluidin blue or neutral red tiny granules are seen in the epidermis and the underlying mesoderm. They are grouped in the Golgi zone and stain metachromatically with toluidin blue, assuming a deep or even bright crimson coloration. They become yellowish with neutral red. After more prolonged staining with toluidin blue very minute granules which stain reddish violet appear *de novo* in the epidermal cells, often as small chains distributed between the bigger granules in the Golgi zone. Although their nature is obscure they appear to be quite distinct from the Golgi substance, as the chains are irregularly distributed in the Golgi zone, whereas the Golgi net is a general cell structure in this phase of development (see also Ries, 1938 for vital staining phenomena in the Golgi zone).

After 1–1½ hr immersion in the toluidin blue solution more regional differences appear in the reaction of the mesoderm to the stain. The cytoplasm of the epidermis and peripheral mesoderm stains bright blue while the granules become deep purple, but the axial tissue, which is destined to form cartilage, hardly ever shows a diffuse cytoplasmic staining. Only the granules in the Golgi zone of those cells (rarely a few granules scattered through the other parts of the cytoplasm) become a brilliant red.

It is probable that here a true metachromatism exists. As the chondrocytes form chromotropic substances such as chondroitin sulphuric acid of the cartilage matrix, it seems very likely that the cells contain such substances in their cytoplasm also.

Regional differences, although less clearly defined, appear too in limb-buds fixed in Champy's fluid and treated to demonstrate the Golgi substance, in

such preparations the cells of the chondrogenic mesoderm reduce osmic acid less than those of the peripheral mesoderm. It is interesting to note that all mesenchyme cells of the limb-bud reduce osmic acid more than the mesoderm cells of the body wall (Fig 1*b*), so that a sharp borderline appears between body and limb-bud. The Golgi complex of the limb-bud also differs from that of the adjacent body wall, in that the strands of the network are much finer than in the latter (Fig 1*a*, *b*).

Ascorbic acid is present in small quantities. It appears evenly distributed in the mesenchyme but for a slight concentration in the anterior part of the base of the limb-bud, which is often difficult to perceive (Fig 8*a*). Glycogen could not be demonstrated in the cells.

(b) The differentiating limb-bud (4-5½ days)

When histological differentiation begins, changes appear in the cytoplasmic inclusions of the different regions. As development proceeds the mitochondria, both in vitally stained and in fixed preparations, appear as rod-like structures, although filamentous forms are also found. When procartilaginous cells develop into young chondroblasts their rod-like mitochondria again lengthen into filaments. Whereas the mitochondria remain irregularly distributed in the mesenchyme cells and the chondroblasts, those of the myoblasts gradually assume a longitudinal orientation.

In preparations vitally stained with toluidin blue some of the mitochondria in all types of cell except (perhaps) those of the epidermis become metachromatically stained. Similar preparations, vitally stained with Janus green B, show many more mitochondria per cell than those treated with toluidin blue.

The Golgi complex shows prominent changes. In procartilaginous cells of the 4-day limb-bud the network becomes smaller than it was in the undifferentiated chondrogenic tissue. Small clear vacuoles which are also visible in the living cell, lie in the meshes of the net (Fig 2). As the development of the cartilage advances the strands break up and shorten, while granular swellings appear in the now thickened Golgi substance. The break-up of the net continues until only short osmophilic rods and granules remain. A distinct difference between the flattened and rounded cells of the diaphyseal, intermediate and epiphyseal regions appears, in the flattened cells (Fig 3) the Golgi net disintegrates more extensively than in the round cells (Fig 4) and becomes reduced to granular osmophilic remnants.

The Golgi net of the mesenchymatous limb-bud cells gradually changes its form to that of the mesodermic cells of the adjacent body wall: the strands shorten and thicken and the distinct but fragile net disappears (Fig 5*a*).

In the myogenic cells also the Golgi net shows a tendency to break up into small fragments and in more advanced stages of development a definite network is seldom distinguishable.

During the early stages of development, as already described, the reducing

power of the cytoplasm towards osmic acid differs in the various regions of the limb-bud. As differentiation progresses, however, the mesenchyme cells gradually lose this power, so that in the $5\frac{1}{2}$ -day bud the cytoplasm of these cells is almost colourless after treatment with osmic acid.

Fat globules can be demonstrated in the Golgi zone of all types of cell both in the untreated living material and in that post-vitally stained with Sudan red or black, or fixed with osmic acid. The fat content is higher in the mesenchyme cells than in the chondroblasts. It appears that the small vacuoles in the Golgi zone of the chondroblasts do not contain fat.

When vitally stained with toluidin blue the $4\frac{1}{2}$ -day limb-bud shows the same reaction as the undifferentiated bud (Fig. 6). Epidermis and mesenchyme appear diffuse blue and each cell contains a few big purple granules and chains of smaller particles. The vacuoles of the Golgi zone do not take up the dye. In the cells of the epiphyseal and diaphyseal zones of the early cartilage similar chains of small granules are seen in the Golgi zone, but occur only very rarely in the flattened cells of the intermediate zone (Fig. 7).

More prolonged staining with toluidin blue produces essentially the same effect as in the undifferentiated limb-bud, the cytoplasm of the epithelial cells and the peripheral mesenchyme stains diffusely blue, but that of the chondroblasts remains colourless.

The changes in the distribution of ascorbic acid during differentiation are interesting. The concentration demonstrated in the anterior basal region of the 3-day limb-bud becomes more and more distinct (Fig. 8*a*) until between the 4th and the 5th day it has formed a ring round the basal part of the bud (Fig. 8*b*). As differentiation advances still further, however, this basal condensation disappears (Fig. 8*c*), but careful microscopic examination shows that the myoblasts contain more ascorbic acid than the mesenchyme cells, and also more than the chondroblasts, which contain very little. At the same time ($5\frac{1}{2}$ days) a new condensation appears in the distal part of the bud (Fig. 8*c*) and soon breaks up into secondary groups lying between the cartilaginous metatarsal rudiments.

The general behaviour and distribution of ascorbic acid in the limb-bud at different stages of development strongly suggest that the local concentrations of this material represent the myogenic regions, but this view required experimental verification (see below).

Since the unincubated egg contains no vitamin C this must be synthesized by the cells, but the black granules seen in the preparations are scattered irregularly in the cytoplasm (Fig. 9) so that there is no evidence as to which part of the cells forms it. Besides the black granules the cells contain brownish granules also which obviously have a lower reducing power and whose nature is unknown.

Again, glycogen fails to show in the cells of the early developing stages. Even the myogenic region lacks it, until true muscle cells develop. In these cells glycogen could be demonstrated for the first time.

*Experiments on the developmental potencies of different regions
of the limb-bud mesoderm*

Although a definite boundary between the myogenic and chondrogenic tissue cannot be demonstrated microscopically in the 3-day bud, it must be fairly sharp. Thus when a series of explants was made, each explant extending a little farther into the interior of the bud, a point was reached at which one explant formed only muscle while another only slightly larger formed both muscle and cartilage. In the undifferentiated distal part of the older limb-buds it would seem that in the tibial region the myogenic and chondrogenic centres of differentiation lie very close together and that the former is very narrow, since it was found impossible to make explants which contained myogenic tissue only, both cartilage and muscle always developed.

To test the view that the tissue with the higher ascorbic acid content was the myogenic region, explants of the ascorbic acid concentration of the 3-day limb-bud were excised and cultivated *in vitro*. By the 10th passage these cultures contained abundant myoblasts. No muscle developed in similar explants of the foot region, however, before the appearance of the distal condensation of ascorbic acid, but when these condensations appeared, i.e. between the 5th and the 6th day, explants of the foot produced muscle *in vitro*.

DISCUSSION

Whilst the surrounding mesenchyme cells readily absorb basic dyes little absorption by chondrogenic cells was observed. Unfortunately it is not yet possible to interpret the difference in absorption of basic dyes in terms of different protein structure.

On the other hand, the presence of metachromatically stained granules in the cytoplasm, especially in that of procartilage cells and chondroblasts, may indicate the presence of sulphuric acid esters of high molecular weight, of which two kinds are known to occur in mesenchymatous structures, viz. mucic acid sulphuric acid in connective tissue-ground substance and chondroitin sulphuric acid in cartilage matrix. It is interesting that the undifferentiated mesenchyme cells which give rise to both connective tissue and cartilage, contain metachromatically stained granules in their cytoplasm, thereby indicating that these substances are not extracellular products. It is possible that the use of mucinase, an enzyme that splits mucic acid sulphuric acid, might allow a further analysis of the observed metachromatic granules.

Whether the stronger reduction of osmic acid by the cytoplasm of procartilage cells is due to a higher content of glucosamine, one of the breakdown products of chondroitin sulphuric acid, needs further investigation. It is interesting to note that a high content of ascorbic acid is only found in cells destined to form muscle tissue and at a stage preceding histological differentiation. It is a striking point that glycogen fails to appear in these cells before they are differentiated into muscle fibres.

SUMMARY

1 The vital staining processes and the changes in the cell structures of the various regions during the early differentiation of the limb-bud of the fowl are described

2 Fats and ascorbic acid could be demonstrated in the cells. Glycogen on the other hand could not be found with the help of various histochemical reactions

3 The experiments with vital staining showed that there must be a physiological differentiation in the limb-bud before any morphological differentiation could be detected. The procartilage cells show no diffuse staining of the cytoplasm, whereas the other cells appear diffusely stained blue with toluidin blue

4 The distribution of ascorbic acid also indicates an early differentiation as it is concentrated in the future muscle cells. Experiments with tissue cultures made it highly probable that differentiation into myoblasts only occurs when the concentration of ascorbic acid in the cells can be demonstrated

5 The changes of the mitochondria and the Golgi complex during the early developmental stages of the limb-bud are described

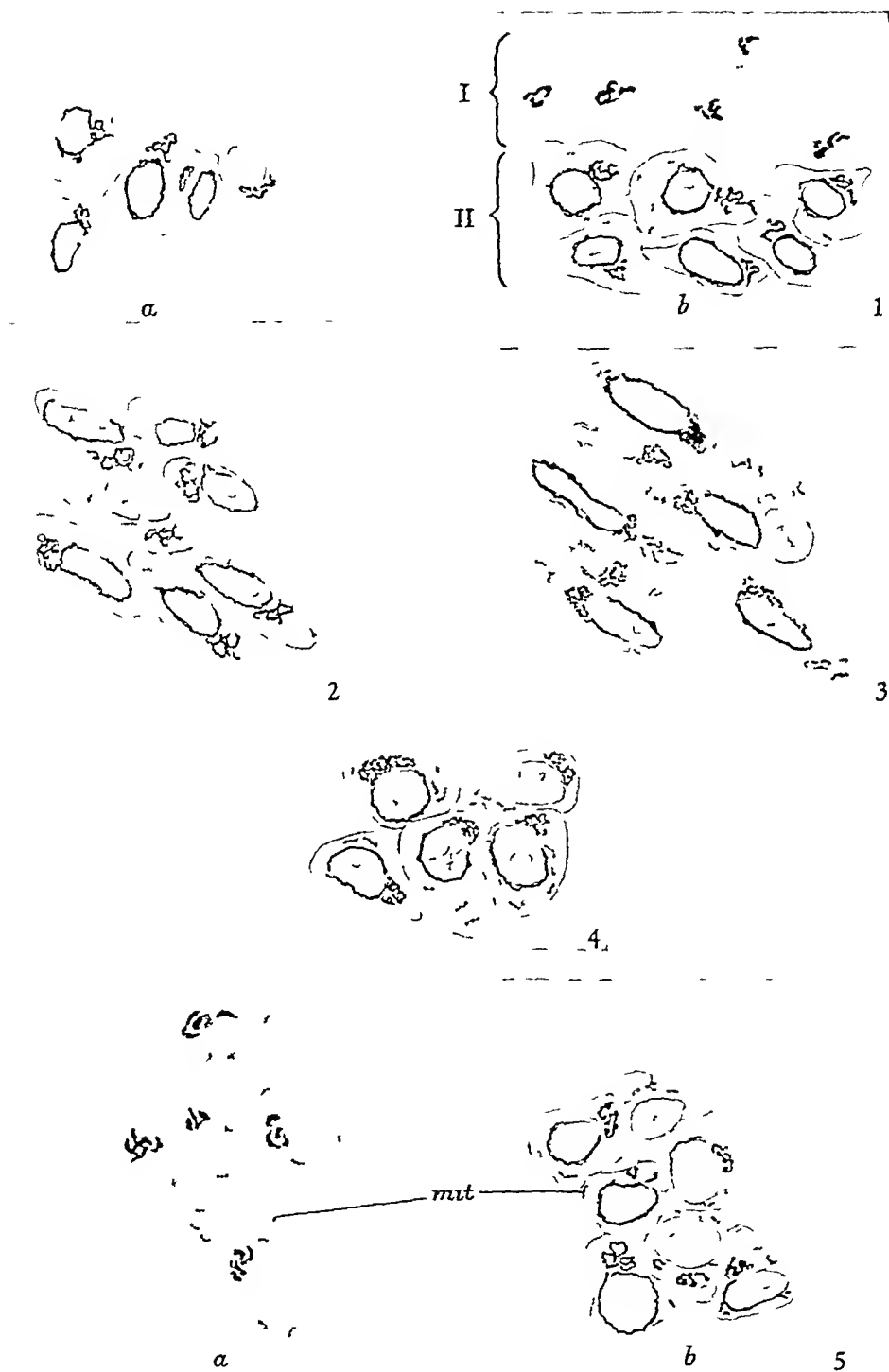
I am indebted to Dr H. B. Fell, Director of the Strangeways Research Laboratory, for his help and advice during the course of the investigations

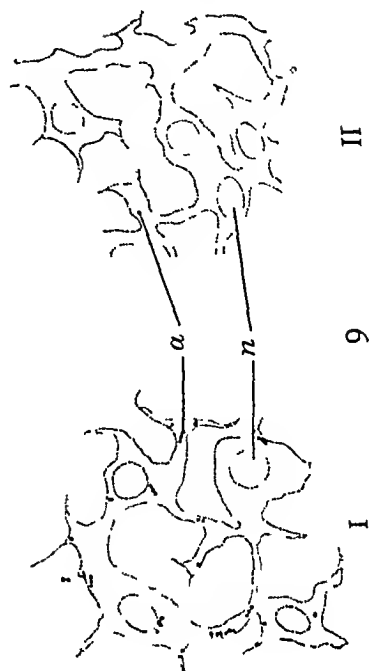
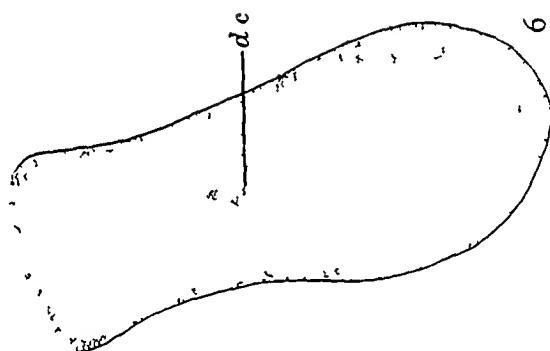
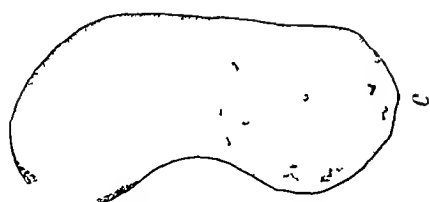
My thanks are also due to Dr W. Jacobson of the same Laboratory, whose suggestions and kind help were very valuable to me

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EXPLANATION OF PLATES

PLATE 1

- Fig 1 Golgi complex in cells of the 3 day bud (a) procartilage cells (b) cells at the border line between body and limb bud I, cells of the body wall, II, mesenchyme cells of the limb bud
Fixative Champy, osmic acid 9 days $\times 900$
- Fig 2 Golgi complex of procartilage $\times 900$
- Fig 3 Golgi complex in flattened cartilage cells of the 5 day bud $\times 900$
- Fig 4 Golgi complex in the rounded cartilage cells of the 5 day bud. $\times 900$
- Fig 5 Golgi complex in mesenchyme (a) and cartilage (b) of the 4-day bud *mit* mitochondria $\times 900$

PLATE 2

- Fig 6 General view of $4\frac{1}{2}$ day bud vitally stained with toluidin blue The degree of staining is represented by the closeness of the stippling Epithelium and mesenchyme diffusely blue, cartilage unstained Note degenerating cells (d c) which stain diffusely deep blue
- Fig 7 Various cells of the 5 day bud, vitally stained with toluidin blue (a) Mesenchyme, (b) rounded, (c) flattened cartilage cells Note the metachromatically stained mitochondria $\times 900$
- Fig 8 Distribution of ascorbic acid in the limb bud Longitudinal sections Dense stippling indicates condensation of ascorbic acid (a) 3 days, (b) $4\frac{1}{2}$ days, (c) $5\frac{1}{2}$ days $\times 20$
- Fig. 9 Distribution of ascorbic acid in the cells of a 3 day bud I, cells of the region of concentration, II, cells of the apex of the limb bud, a ascorbic acid, n nucleus $\times 800$

THE COMPARATIVE ANATOMY OF THE INGUINAL LIGAMENT

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INTRODUCTION

Since the description of the inguinal ligament by Fallopius (1562) there has been much uncertainty regarding its nature, importance and function in Man. The name of Poupart (1705) is remembered more for his brief communication on this subject than for all his surgical attainment. Haller (1774) realized that Poupart had referred to 'ligamentum Fallopii pro re nova'. Macalister (1899) has given a full account of this early history.

In 1793, Gimbernat added his description of the pectineal part of the inguinal ligament and is known to have demonstrated it to John Hunter while visiting London. Cloquet (1817) referred to Gimbernat's ligament as a term in current use in England. Astley Cooper (1827, 1830), in his treatises on the testis and on hernia, gave accounts of his 'ligament of the pubis', now known as the pectineal ligament.

Parsons (1903) doubted whether an inguinal ligament was present in mammals other than Man and considered that 'in the lower mammals Poupart's ligament as a definite structure can hardly be said to exist'. Following the comparisons made by Keith (1894, 1921) between Man and other Primates, current opinion inclines to the view that the inguinal ligament is peculiar to Man and that the mechanism of the groin has been fundamentally changed from that found in other animals in order to meet the demands of the erect posture. In a recent paper on this subject Miller (1947) draws attention to these 'fundamental differences' yet also finds that the inguinal canal of Man is 'fundamentally of the same plan as in other Primates'.

The purpose of the present paper is to look beyond the Primates and to see whether any basic principles can be found within the animal kingdom which will assist in the understanding of the human groin and in the dispersal of any doubts and errors that exist.

MATERIAL AND METHOD

The animals listed below have been examined in order to ascertain the disposition of the structures in the region of the groin. Special reference has been made to the attachments of the abdominal muscles and to fascial condensations which merit description as distinct structures.

Dissection and measurement of most of this material was commenced before fixation. The distances between bony points were found to be determined with greater accuracy while the overlying soft tissues were fresh. At the same time

the transparent undifferentiated connective tissue was distinguished more easily from such tissue that had been rendered opaque by the condensation of collagen. Dissection was completed after fixation in 5% formalin. In a few instances material was taken from the stores of the Royal College of Surgeons of England. The remainder was obtained from the Zoological Society of London.

The following animals have been dissected

(Nomenclature in accordance with the *List of the Vertebrated Animals exhibited in the Gardens of the Zoological Society of London* Vol. I, London, 1929)

REPTILIA	—	<i>Crocodylus palustris</i>
MONOTREMATA	—	<i>Ornithorhynchus paradoxus</i>
MARSUPIALIA	—	<i>Macropus ualabatus</i> (2)
XENARTHRA	—	<i>Choloepus didactylus</i>
ARTIODACTYLA	BOVIDÆ	<i>Anoa depressicornis</i> <i>Connochaetes gnu</i> <i>Cephalophus dorsalis</i>
	GIRAFFIDÆ	<i>Giraffa reticulata</i>
	—	<i>Procavia capensis</i>
HYRACOIDEA	—	
RODENTIA	SCIURIDÆ	<i>Euxerus erythropus</i>
	MYOCASTORIDÆ	<i>Myocaster coypus</i>
	CAVIDÆ	<i>Dolichotis magellanicus</i>
PINNIPEDIA	—	<i>Zalophus californianus</i>
CARNIVORA	FELIDÆ	<i>Acinonyx jubatus</i>
	CANIDÆ	<i>Lycan pictus</i>
	PROCYONIDÆ	<i>Procyon lotor</i>
	—	<i>Nasua nasua</i>
CHIROPTERA	—	<i>Pteropus medius</i>
LIPOTYPHILA	—	<i>Erinaceus europæus</i>
MENOTYPHILA	—	<i>Tupaia insignis</i>
PRIMATES	CERCOPITHECIDÆ	<i>Colobus polykomos</i> <i>Cercopithecus mona</i> <i>C. nictitans</i> (2) <i>C. aethiops</i> <i>C. tatalus</i> (2)
		<i>Gorilla gorilla</i>
	PONGIDÆ	

Reptiles

In quadrupedal reptiles (e.g. crocodile, Fig. 1) it is possible to distinguish a tendinous arch over the psoas muscle (*E*) and femoral vessels (*F*) as they pass behind the abdominal muscles on their way to the thigh. There are also other arches over the origins of the muscles of the adductor group. The lower fibres of the internal oblique (*C*) and transversus (*D*) take attachment from these arches and sweep towards the mid-line. The external oblique (*B*) has a muscular distal border which blends with these arches deeply and is attached superficially to the skin crease of the groin.

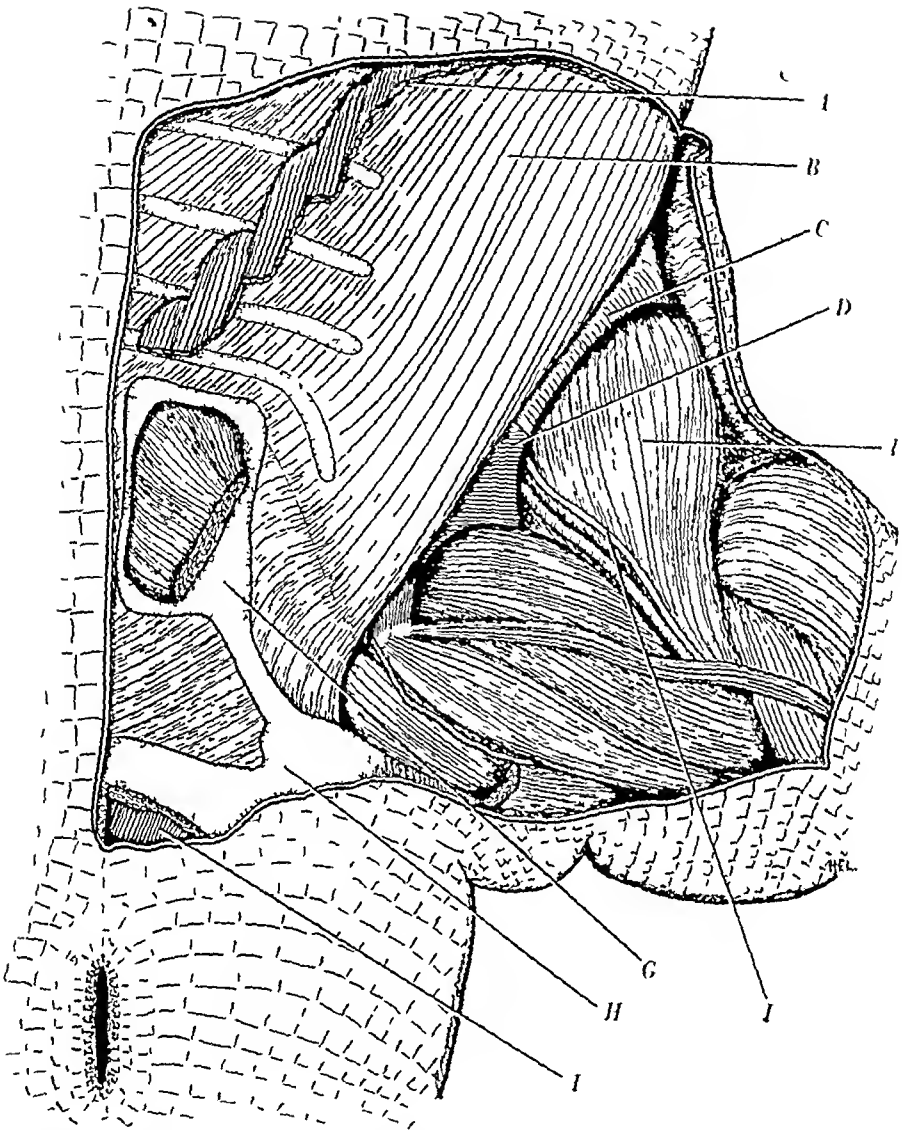


Fig 1 The groin of a crocodile (*Crocodylus palustris*) (A) The rectus abdominis, cut through its proximal fibres (B) The external oblique which has been retracted medially to display the hylum of the hind limb (C) The internal oblique attached to a thin but distinct crural arch (D) The transversus abdominis with a similar attachment for its lower fibres (E) The psoas (F) The femoral nerve (and artery) (G) The epipubic cartilage (H) The pubic bone (I) Part of the distal attachment of the rectus abdominis

Mammalian pronograde quadrupeds

(1) *Muscular attachments* The anatomy of the mammalian groin is found to follow the principles seen in the reptiles. The fibrous arch over the psoas muscle is a constant feature of mammalian anatomy and serves as the attachment for the lower fibres of the internal oblique. It was known to the older anatomists, Gimbernat (1793) and Astley Cooper (1827), as the crural arch. The transversus abdominis is attached in part to this arch and in part to the fascia covering

ilio-psoas (to the tendon of the psoas minor when present) These basic attachments to the deep component of the inguinal ligament are constant whether the external oblique contributes to it or not

When the external oblique consists of muscular fibres at its distal border, these fibres are attached to the underlying internal oblique muscle by loose

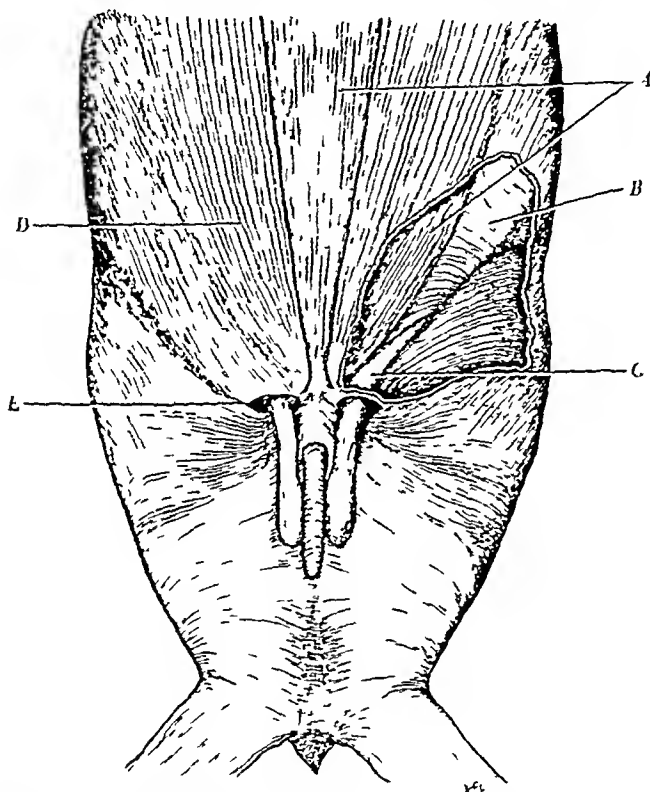


Fig 2 Dissection of the abdominal muscles of a sea lion (*Zalophus californianus*) (A) The rectus abdominis overlapped in part by fibres of the external oblique (B) The internal oblique found, after removal of the overlying external oblique fibres, to be attached to the crural arch (C) The cremaster, the fibres of which can be traced upwards to be continuous with those of the transversus abdominis (D) The muscle fibres of external oblique are disposed as a thin flat sheet which sweeps distally over the groin and blends with the deep fascia of the thigh. There is no aponeurosis of the external oblique in this case (E) The superficial inguinal ring through which the spermatic cord passes on its way to the subcutaneous pouch in which the testes are lodged

areolar tissue In such a case (sea-lion, Fig 2) only the deeper components of the inguinal ligament are present and the external oblique does not contribute to it

When the distal border of the external oblique is aponeurotic, it arches over the psoas muscle and the femoral vessels, blending deeply with the crural arch

and passing medially to its attachment to the superior surface of the pubis. Further distally the aponeurosis becomes continuous with the deep fascia of the thigh. This may conceal the thickening in the inturned margin of the aponeurosis which, although never so well marked as in Man, is sufficient to indicate that a true inguinal ligament is present. The aponeurosis is adherent to the underlying lower fibres of the internal oblique but not so firmly that

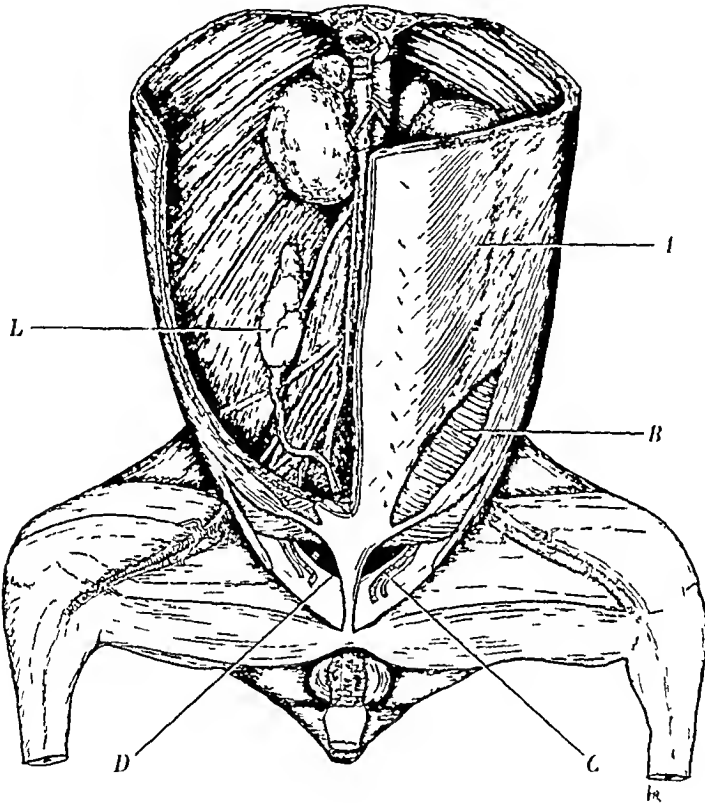


Fig 3 Dissection of a hyrax (*Procavia habessinica*) (A) The external oblique which passes into an aponeurosis near the mid line anteriorly, and also along its distal border which is inturned. The distal three digitations are widely separated from each other when the thighs are abducted, and through the most distal of these intervals large vessels and a nerve pass into the skin of the groin (B) The internal oblique seen between two of the digitations of external oblique, with a characteristically arched lower border (C) Vessels and a nerve passing into the skin of the groin (D) The thickened, inturned edge of the aponeurosis of the external oblique (E) The testis and epididymis which are permanently intra abdominal, and which have played no part in the disposition of the structures described above

such fibres could be said to arise from the inguinal ligament. No detail of this arrangement can be held to have resulted from the descent of the testis. The same general description applies both to testiconda (e.g. hyrax, Fig 3) and to those animals in which the testes descend (e.g. cavy, Fig 4).

(2) *Comparative measurements* The two inguinal ligaments subtend between them a smaller angle in the pronograde quadruped than in Man, the average measurements being approximately 60 and 90° respectively. Thus in quad-

rupts the direction of the inguinal ligament is almost the same as that of the lateral border of the rectus abdominis, which thus gives support to the inguinal canal in those forms in which testicular descent occurs

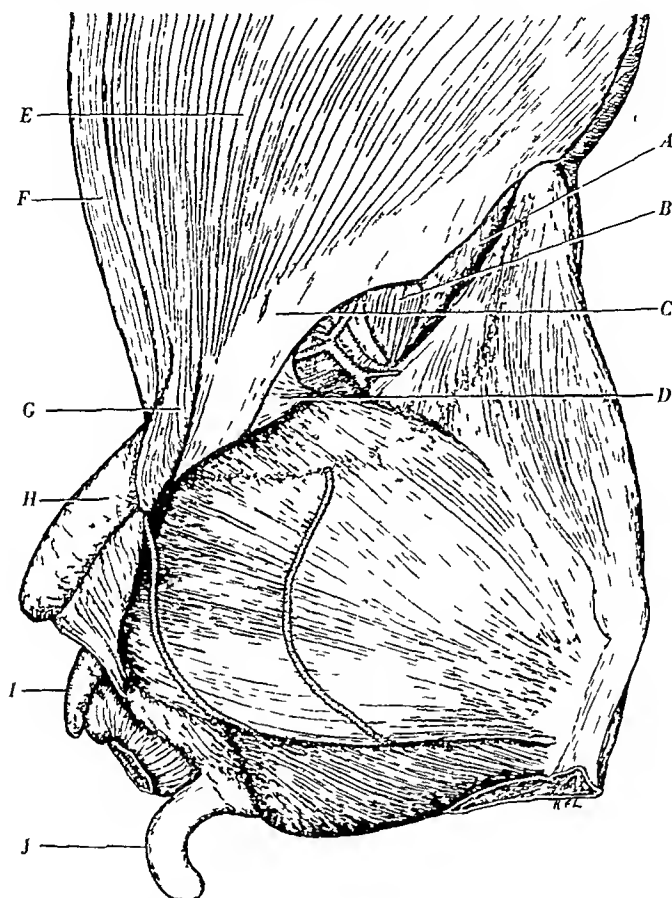


Fig 4 Lateral view of the groin of a Patagonian cavy (*Dolichotis magellanica*) The left thigh has been widely abducted and the external oblique has been drawn proximally away from the inner surface of the thigh with which it is normally in contact (A) Thickening in the psoas fascia (B) The psoas (C) The aponeurosis of the external oblique freed from the fascia lata and retracted proximally (D) The pectineal part of the inguinal ligament (E) The external oblique muscular part (F) The external oblique, central tendinous part (G) Muscular fibres forming the superficial inguinal ring (H) The testis enclosed in the external spermatic fascia (I) The penis (J) The tail

The length of the human inguinal ligament is usually measured from the anterior superior iliac spine to the pubic tubercle (e.g. Panton, 1929). As it is rarely possible to find a point homologous with the pubic tubercle in mammals other than Man, the distance between the anterior superior iliac spine and the

inguinal ligament gives attachment to the cremaster muscle from its concave proximal surface and is loosely adherent distally to the reticular deep fascia over pectineus

CONCLUSIONS

A study of the mammalian groin reveals the presence of ligamentous connective tissue in a plane ventral to the pelvic girdle. Despite previous doubts and denials, such tissue has been identified by the writer in each of the species examined. Structures homologous with the deep components of the inguinal ligament of Man, the ilio-pectineal ligament and the deep crural arch, have been found to exist in all the other mammals dissected. They may thus be described as the basic components of the mammalian inguinal ligament.

A contribution from the external oblique is added to these basic structures when this muscle is aponeurotic near its inferior margin. The pectineal part of the inguinal ligament (Gimbernat's ligament) can be identified in those mammals which have such a contribution from the external oblique aponeurosis. Anson & McVay (1940) have strongly contested the current description of the inguinal musculature taking origin from the internal lower border of the external oblique aponeurosis. The results of this comparative investigation will lend support to the conclusions of these workers.

SUMMARY

- 1 Reference is made to previous descriptions of the inguinal ligament.
- 2 The anatomical principles that underlie the formation of this ligament are discussed.
- 3 An inguinal ligament is found to be a constant feature of mammalian anatomy.
- 4 The external oblique aponeurosis, although a major component of the inguinal ligament in Man, is of variable importance in other forms.
- 5 A fibro-tendinous arch over the ilio-psoas muscle and the femoral vessels is a constant basic component of the inguinal ligament in all animals with hind limbs.
- 6 The current description of the inguinal ligament in man is not compatible with the basic principles found to underlie the structure of the mammalian groin.
- 7 The description of the inguinal region given by Anson & McVay (1940) is corroborated.

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I am indebted to the Zoological Society and to Dr R. E. Rewell for the supply of the material from which most of the dissections were made.

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OBSERVATIONS ON THE INNERVATION OF BLOOD VESSELS

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Some of the earliest observations on the association between the sympathetic nervous system and the blood vessels were made by anatomists (Bichat, 1802). Nevertheless, despite the obvious importance of an exact knowledge of the nerve supply of the blood vessels in the interpretation of peripheral vascular reactions, anatomical descriptions of the pattern of vascular innervation have been very conflicting.

The physiological researches of Langley (1921), Bayliss (1923) and others, and the results obtained from surgery of the autonomic nervous system (Leriche, 1913, White & Smithwick, 1941) have added much to our knowledge of the action of the vaso-motor nerves, the pathways traversed by them, and the part played by chemical substances liberated at the nerve endings on the blood vessels. The early recovery of tone in blood vessels following sympathectomy indicates that the problems concerning the regulation of the blood flow in peripheral vessels are not completely solved. Freeman, Smithwick & White (1934) have brought forward evidence to show that this return of tone is related to an increased sensitization of the smooth muscle in the wall of the vessels to chemical substances circulating in the blood stream. Their conclusions, however, take no account of the possible role played by the large number of nerve fibres that persist in the walls of the peripheral blood vessels after sympathectomy (Hinsey, 1929, Woollard, Weddell & Harpman, 1940).

Most of the anatomical descriptions have been derived from the study of histological preparations following silver impregnation, and the confused state of present-day knowledge is well exemplified in the accounts given by Stohr (1935) and Boeke (1940). These authors tend to use extreme magnifications and they and others describe minute nerve fibres entering endless ground plexuses and periterminal networks (see Nonidez 1936, for discussion). The maze of nerve fibres that they portray seems to be arranged in no particular pattern and it is difficult to see how such a framework can provide an anatomical basis for the physiological reactions which occur.

In striking contrast to the descriptions based on the examination of silver preparations are those given by Woollard (1926) who used cleared whole specimens stained intra-vitally with methylene blue. Woollard, from the results of his investigations, was able to show that the manner of innervation of the blood vessels provides a reasonable morphological basis for physiological reactions. In his account, however, he does not draw any distinction between the elaborate arrangement of plexuses for the supply of the small arteries

* Work carried out during leave of absence from Queen's University, Belfast

and the simpler network that supplies the arterioles and pre-capillaries. The illustrations to his article, made from drawings, err in the direction of oversimplification and do not convey a clear representation of all the conditions found on the microscopic examination of methylene blue preparations.

In the present paper the pattern of innervation of the smaller blood vessels in the stomach and in the ear of the rabbit is described, and a point has been made of illustrating the findings photographically. It will be seen that, in general, the description given approximates that of Woollard. However, the examination of better preparations has made it possible to trace the nerve fibres farther towards their terminations and to add further details of the pattern of innervation. An attempt has been made also to interpret some of the known physiological reactions in terms of the anatomical findings. Further, in the course of the investigation, observations have been made which suggest that some of the fibres described as nerves by many of those employing silver techniques are probably connective tissue fibres.

MATERIAL AND METHODS

The blood vessels studied were situated in the ear and in the stomach wall of young rabbits of weight 1.5-2.5 kg, and their innervation was studied by utilizing the staining properties of methylene blue. The animals formed part of a series used by Feindel, Sinclair & Weddell in experiments designed to develop an intravenous methylene blue staining technique for the investigation of the nervous system. The preparations upon which the present paper was based represent some of the first successful results obtained. It should therefore be noted that the technique here reported, although satisfactory in these particular instances, is not consistent. A more reliable staining method has subsequently been developed as a result of the continuation of these experiments and is now in the course of publication (Feindel, Sinclair & Weddell, 1948).

For the purpose of record, however, it should be stated that the animals reported in this paper were injected in the following manner. From 20 to 30 c.c. of a 0.5% solution of methylene blue (B.D.H.) in 0.25% sodium citrate solution were injected by slow intravenous drip into the anaesthetized animal. Following this the thorax was opened and a further injection of about 20 c.c. of methylene blue was made directly into the left ventricle under a pressure of approximately 200 mm Hg.

By opening the abdomen the stomach was exposed to the air for about 20 min. to allow blueing to occur. The serous membrane was first stripped from the muscular wall after washing. The subsequent treatment of the tissues concerned followed the technique described by Weddell & Glees (1941).

HISTOLOGICAL OBSERVATIONS

In the present work, neither the veins nor the larger arteries were examined but there were seen in the preparations contributions from the regional nerve trunks to the intramural plexuses of the arteries as described by numerous

authors (Kramer & Todd, 1914, Blair, Duff & Bingham, 1930, Woollard & Phillips, 1932)

During the examination of methylene blue preparations from the stomach and ear of the rabbit it was found that there was sometimes incidental staining of the nuclei, and occasionally even of the cytoplasm, of some of the smooth muscle fibres. This circumstance made it much easier to determine the exact relationship of the nerve fibres to the different coats of the vessel. In addition it was noted that the fibrous and reticular connective tissue present in the subcutaneous layer of the ear and the subserous layer of the stomach was sometimes well stained. In Pl 1, fig 1 there are shown two nerve fibre fasciculi in the subcutaneous tissue of a rabbit's ear, crossing a background of stained reticular fibres. It is apparent from this photograph that, on first inspection, the reticular connective tissue strands might be mistaken for nerve fibres, but a careful examination of large pieces of tissue makes it possible to distinguish with certainty these strands from true nerve fibres.

In the tunica adventitia of the small arteries there may be seen nerve fibres having a general longitudinal direction. The arrangement of these fibres, in a small artery of the stomach wall, is depicted in Pl 1, fig 2 which shows portions of five fibres running in the long axis of the vessel. Situated more deeply and lying transversely there can be seen, as dark striations, a few smooth muscle nuclei that have taken up the methylene blue stain. The fibre marked *A*, after running for a considerable distance, turns sideways and divides into two branches, one of which pursues a recurrent course while the other continues the direction of the original fibre. Both from the main fibre and from its recurrent branch are given off branches (four or five in number) which are slightly out of focus, being more deeply placed, and which have a beaded appearance. The fibre marked *B* runs, within the limits of the photograph, for only a short distance before dividing into two (or three) branches which in their turn soon break up into further branches. These branches, which present an irregular appearance, are placed between the parent fibre and the nuclei of the smooth muscle cells and are, therefore, not sharply in focus. Fibre *C* runs the whole length of the field without giving off any large branches and its superficial position in relation to the stained nuclei is well shown. Another fibre, *D*, crosses this latter fibre and then terminates in the tunica adventitia about the middle of the field in an ending which may be of a sensory type. The preparation was not, however, clear enough to allow its nature to be accurately determined.

It is difficult to decide whether the fibres that have been here described are finely medullated or non-medullated, for, using the methylene blue technique, it is considered permissible to designate fibres as medullated only when nodes of Ranvier can be clearly distinguished. It is quite certain, however, that in this outer adventitial plexus both finely medullated and non-medullated fibres are present, but for the reasons given the relative numbers of either variety cannot be stated.

Pl 1, fig 3 shows the intricate plexus of varicose nerve fibres lying on the outer surface of the tunica media of a small artery. The fibres divide and interweave with one another in an exceedingly complex fashion, giving the appearance of a net with coarse meshes. In some instances the nerve fibres forming the strands of the mesh do in fact form complete loops and communicate with one another. The meshes of the network are irregular in outline. The smooth muscle nuclei, for some reason, are not stained but the transverse striations in the background indicate the direction of the fibres of the tunica media. A single fibre from the adventitial plexus just described can be seen dividing into two branches which join the plexus. Just outside the limits of the field shown, another fibre, also from the adventitial plexus, could be seen behaving in a similar manner.

Lying within the tunica media of the small arteries is a plexus of fine beaded fibres. These fibres are seen in Pl 1, fig 4, forming a regular zig-zag pattern. Some of the muscle nuclei, only partly shown, are darkly stained and lie nearly parallel to one another. Exceedingly fine branches of this plexus can be traced, some appear to end freely in the tunica media while others show knob-like endings in close relation to the nuclei of the smooth muscle cells. One such ending is shown under high magnification in Pl 1, fig 5. The nucleus of the fibre appears as a dark oval and the stained cytoplasm can also be faintly seen. A short segment of a very beaded fibre can be seen approaching the nucleus as a series of moniliform expansions almost at right angles to it and one knob lies just above the nucleus. The nuclei of other muscle fibres can also be seen in the illustration as faint parallel shadows.

An organized ending, probably of a sensory type, is shown in Pl 1, fig 6, lying in the tunica adventitia of a small artery. This ending is composed of a number of short thick branches and is borne by a medullated fibre which is not quite in focus in the photograph. In the same field another medullated fibre can be seen and the blurred dark oval shadows are transversely-placed stained nuclei of the smooth muscle fibres of the tunica media.

Pl 2, fig 7, shows another medullated fibre lying among fat cells close to the wall of a small vessel. The position of the vessel is indicated by transversely-placed muscle fibres which can be recognized because in some of them the nuclei and cytoplasm have taken up the dye. The nerve fibre makes a loop as it leaves the artery and then disappears among the fat cells whose dark nuclei and faintly stained cell walls make them readily distinguishable.

A general picture of the innervation of an arteriole is seen in Pl 2, fig 8. A fasciculus composed of about seventeen nerve fibres meanders across the field and in the portion shown in the figure it gives off four smaller bundles of from four to nine fibres and three single fibres. The arteriole, identifiable by occasional stained nuclei, runs near the main fasciculus and in one place can be seen crossing superficial to one of its loops. Most of the branches of the tract do not appear to be in any way concerned with the innervation of the vessel and were traced for varying distances into the surrounding muscle before

being lost A single fibre, *A*, leaves the main trunk close to the arteriole, makes a wide loop, crosses the vessel, and then runs near to it before finally joining the nerve fibres lying directly on its wall The nerve fibres on the arteriole itself are difficult to distinguish in this picture but are better shown in Pl 2, fig 9, an enlargement of a small portion of the vessel depicted in Pl 2, fig 8 Pl 2, fig 9 shows two fine beaded nerve fibres placed one on either side of the arteriole in which four muscle cells are stained These fibres appear to lie directly on the wall of the vessel, and running parallel to the vessel close to it there are two small nerve fibres On the left of the field the main fasciculus can be seen and branching from it a smaller bundle in which two medullated fibres can be distinguished A single fibre whose Schwann sheath for some reason has been stained by the methylene blue makes several small loops and runs for a short distance close to the vessel wall before passing deep to it

The more detailed innervation of an arteriole is shown in Pl 2, fig 10 Running in the long axis of the vessel and lying, it appears, directly on the muscle wall and placed one on either side of the vessel there are two fibres showing well-marked irregularities Branches from these fibres can be seen crossing the vessel and forming a loose network on its surface In this vessel the nuclei of the smooth muscle fibres are not stained Pl 2, fig 11 shows a slightly smaller arteriole in which the muscle nuclei are well stained On one side of this vessel can be seen a single nerve fibre which at one place divides and sends a branch across the arteriole From the opposite side a pre-capillary arteriole is given off accompanied by a single fine beaded nerve fibre, and on the same side of the arteriole can be seen, in part, another nerve fibre at a short distance from the main vessel In the arterioles only one plexus of nerve fibres could be found and no actual endings were observed in their walls

Pl 2, fig 12 shows a single fine beaded nerve fibre running on the wall of a capillary blood vessel which is lying beside a large nerve trunk containing some medullated fibres It is difficult to define the wall of the capillary, but its course is sufficiently indicated by the stained nuclei of the cells associated with it No endings were seen on the capillaries nor was there any indication of a plexus formation, indeed, in many of the capillaries studied, the nerve fibres were less closely associated with the vessel than in the one shown

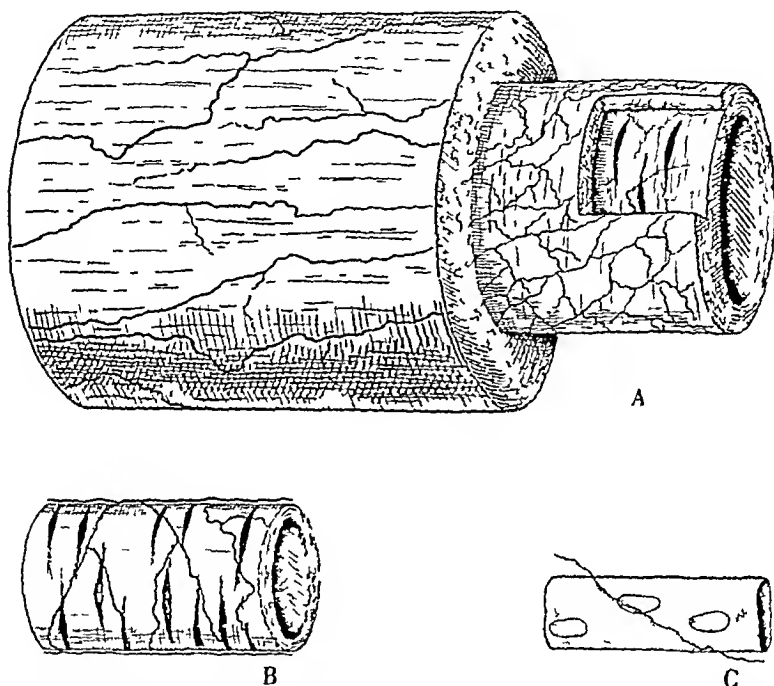
Text-fig 1 is a composite drawing showing the author's conception of the arrangement of the innervation of a small artery, an arteriole, and a capillary, based on the observations that have been made The histological findings may be summarized as follows

Small arteries In the small arteries of the stomach wall the nerve fibres form three plexuses

(a) In the tunica adventitia, nerve fibres run in the long axis of the vessel in roughly parallel formations These fibres branch and send fibres to the deeper plexuses, but there is no indication of a true network The diameter of the fibres is fairly uniform and nodes of Ranvier can sometimes be seen

It is considered, therefore, that this plexus is composed of non-medullated and finely medullated nerve fibres

(b) Between the tunica adventitia and the muscular coat there is a meshwork of non-medullated fibres. The nerve fibres that compose this plexus are irregular in diameter and show many varicosities. There is a complete absence of any regular arrangement of the fibres in a longitudinal or transverse direction, they divide and interweave to form a meshwork of communicating fibres.



Text-fig 1 Drawings to illustrate the innervation of small blood vessels. A, small artery to the left, the plexus in the tunica adventitia is shown. To the right, the plexus between the tunica adventitia and media is seen and also (in a small section) the plexus of fine beaded fibres within the tunica media. B, arteriole, C, capillary.

(c) In the tunica media very fine beaded fibres are present between the smooth muscle cells. A regular pattern is formed by the branching of these fibres, and numerous endings are present. Some of the fibres end as knobs in close relation to the nuclei of the smooth muscle cells, while others appear to terminate freely between the nuclei. It is by no means certain that these latter endings are unrelated to nuclei of muscle fibres since the staining of the muscle cells by methylene blue is quite capricious and may only pick out occasional nuclei here and there.

Arterioles In the arterioles a much simpler arrangement is present. Running alongside the vessels there are nerve fibres which send communicating branches across the vessel. These branches may be few in number or in the larger arterioles sufficiently numerous to form a loose meshwork. The number of fibres accompanying the vessel varies, usually there are two fibres which appear to lie one on either side of the vessel, but in the larger vessels there may be, on one or both sides, a small fasciculus or two or three fibres. In the pre-capillary vessels there is frequently a single fibre which crosses over the vessel at irregular intervals. In none of the material examined were fibres observed to end *directly* on the smooth muscle fibres of the arterioles.

Capillaries Single beaded fibres accompany the capillary blood vessels. These fibres wander from side to side and apparently have only a very general relationship to the wall of the vessel. No endings of any kind were found on the walls of the capillaries.

Sensory endings In association with the blood vessels of the stomach two types of sensory ending are found, both subserved by fine medullated fibres. Medullated fibres are seen ramifying among the fat cells that constantly accompany the vessels and that are, indeed, at times a guide to their location. These fibres can be followed for long distances among the fat cells, and, becoming exceedingly fine, they appear to terminate freely between the cells. A second type of sensory ending, of an organized type, is also found in the tunica adventitia (Pl 2, fig 7).

Classification of the small blood vessels The observations described suggest a classification of blood vessels on the basis of their innervation, viz small (or medium) *arteries*, vessels showing outer and inner adventitial plexuses in addition to a plexus in the tunica media, *arterioles*, vessels having only a single plexus formed by one or two nerve fibres with connecting branches, *pre-capillary arterioles*, smaller vessels having some muscle fibres and, usually, a single nerve fibre, *capillaries*, the smallest vessels, with no muscle nuclei and each commonly having one very finely beaded nerve fibre close to its wall or at a short distance from it.

DISCUSSION

The presence of sympathetic fibres has been established in the blood vessels of the skin (Trotter & Davies, 1909), pia mater and brain (Huber, 1899, Penfield, 1932), skeletal muscle (Hinsey, 1929, Gilding, 1932, Baicroft, Bonnar, Edholm & Effron, 1943), and visceral muscle (Stolir, 1930). It is also well recognized that these fibres subserve vaso-constrictor functions, and there has now accumulated a considerable number of observations which indicate that the blood vessels are supplied with sensory endings, and that active vasodilatation (as distinct from the removal of vaso-constrictor impulses) can occur.

* The anatomical arrangement of the nerve fibres that perform these functions is, however, less well established, and the pathways along which some of the impulses are conveyed are by no means certain.

Several authors have shown that sympathetic nerve fibres can reach the peripheral blood vessels either by direct continuity along the vessels from the aortic plexuses, or by branches given off from regional nerves lying in close relation to the peripheral vessels. It is well known that nerve trunks related to the blood vessels in the limbs or body-wall contribute at intervals branches to their supply. The present investigation has shown that this also holds good for visceral blood vessels.

The arrangement of the nerve fibres in the walls of the blood vessels and their function are less certainly defined. Huber (1899) in the vessels of the pia mater, describes a primary plexus in the tunica adventitia, and a second just external to the muscle coat from which fibres are given off to end in the muscular tissues of the vessels. Stohr (1932) also describes an outer and inner adventitial plexus although the fibres that he illustrates on page 385 of *Cytology and Cellular Pathology of the Nervous System* as forming the inner plexus are patently not nerve fibres. The present work agrees largely with these descriptions but confirms Woollard's (1926) account of a third plexus situated within the tunica media and consisting of fine beaded fibres having endings directly related to the nuclei of the smooth muscle fibres. The existence of this plexus in the tunica media was originally denied by Stohr (1932) who stated that he had never seen fibres actually in the media or ending on the muscle fibres.

Stohr (1935) has, however, subsequently revised his views on the existence of a plexus of nerve fibres within the tunica media and has followed Boeke (1933) and Reiser (1932, 1933) in describing a periterminal network of sympathetic fibres permeating all the tissues of the body, including the media of the blood vessels. Nonidez (1937) has dealt at length with this periterminal network and has shown that the fibres are reticular connective tissue fibres which have been believed to be nervous because of the faulty use of silver impregnation methods. Weddell, Harpman, Lambley & Young (1940), in a paper on the innervation of the tongue, have described the staining of reticular connective tissue fibres in methylene blue preparations and have suggested that high concentration of the dye may be a causative factor. It is sufficient for the present paper to draw attention once again to the advantage of the methylene blue technique, which permits of the use of whole cleared specimens in which nerve fibres can be traced for considerable distances and clearly distinguished from stained reticular strands, thus avoiding the difficulty of interpretation and the greater risk of error associated with the use of silver techniques and serial sections.

The nerves accompanying the arterioles have a much more generalized relationship to the individual muscle fibres. The bulb-like endings on the smooth muscle cells, described by Langworthy & Ortega (1943), have not been observed. The innervation of the capillary bed is apparently still more generalized and a single nerve fibre runs for long distances without reinforcement close to the vessels but not, it would appear, necessarily in contact with the capillary wall. We are not able to say whether such fibres are functionally related to true capillaries, rather than to pre-capillaries or the 'metarterioles' of Chambers

& Zweifach (*vide infra*) Where they course alongside a true capillary, they may be following the vessel to the pre-capillary which feeds it. Our preparations are not adequate to determine this point, for (as already noted) the methylene blue only stains muscle cells occasionally and incidentally.

These observations suggest that the terminals derived from a single fibre may supply a relatively short segment of a small artery with a dense and intimate innervation, a longer segment of an arteriole with a less intimate supply, and a still greater length of capillary vessel with a loose investment of nerve fibres. Proof of this supposition is of course lacking, and much experimental work remains to be done before it can be established. It is, however, interesting to note that spasm of short segments of large arteries has been observed following injury to the vessel (Lewis, 1946).

The present classifications of the blood vessels are unsatisfactory and often conflicting. Lewis (1927) has classified the small blood vessels of the skin into strong arterioles, minute vessels and deep veins. In the category of strong arterioles he includes the main cutaneous arteries, the cutaneous arterial networks and the arched arterioles. The terminal arterioles, capillaries and collecting venules are classified as minute vessels. Reference to the standard text-books of histology shows that the descriptions of the smaller blood vessels are unsatisfactory. Some authors divide the small vessels on the arterial side into small arteries and arterioles, others describe medium arteries and small arteries or arterioles, while pre-capillary arterioles are also recognized by some but not by others (Bloom, 1947, Jordan, 1947, Smith & Copenhagen, 1944, Carleton, 1938). In the most recent study of the organization of the capillary circulation, Chambers & Zweifach (1946) distinguished an element in the capillary bed which they term the 'central channel'. The proximal part of this, the 'metarteriole', is provided with muscle and gives off pre-capillaries which feed the true capillaries.

Woollard (1926) has stated that many of the fibres conveyed to the blood vessels by the peripheral nerves are medullated and has noted the persistence of such fibres in the tunica adventitia following ganglionectomy. Woollard *et al* (1940) have also noted the persistence after sympathetic neurectomy of at least the greater part of the plexus of fibres that lies just external to the tunica media. There is some evidence that these medullated fibres convey afferent impulses and that these impulses pass into the posterior nerve roots, for it is known that these fibres remain following ganglionectomy and section of the ventral nerve roots (Hinsey, 1929). Although it is now generally accepted that active vasodilatation can occur, the nerves concerned in its production have not so far been determined. Barron (1946) holds that vasodilator fibres reach the blood vessels by way of the parasympathetic outflow (in certain parts of the body), by efferent fibres in the sympathetic system, and via the dorsal spinal roots to the blood vessels of the skin. He finds difficulty in accepting the view that impulses conveyed antidromically to the peripheral termination of sensory fibres can affect the blood vessels. Doupe (1943), however, believes

that vaso-dilatation in the skin is due to the formation of metabolites by afferent nerve fibres running in close association with the terminal vessels, and it seems that a possible mechanism for the production of these metabolites may be provided by the medullated fibres in the tunica adventitia and the meshwork of fibres immediately external to the tunica media

Bazett & McGlone (1928), Waterston (1934) and others have elicited pain from the tunica adventitia of arteries, and it has also been shown that some of the medullated fibres accompanying the blood vessels end in Pacinian corpuscles (Rachmanow, 1901, Lawrentjew, 1926, Woollard, 1926) Other medullated fibres serve organized endings in the tunica adventitia (Dogiel, 1898), or end freely between fat cells and in the adventitia itself The presence of free endings among the fat cells and of organized endings in the adventitia has been confirmed in the present investigation for the blood vessels of the stomach It is now generally accepted that in the tunica adventitia of some of the large blood vessels there are receptors which are sensitive to tension changes in the vessels, and it is possible that the free nerve endings among the fat cells are affected by pressure changes in the vessel wall transmitted by the liquid inside the cells The organized endings are not unlike the endings found at the base of the tongue which Weddell *et al* (1940) consider to be of tension-recording type The neural elements subserving the sensation of pain that can be produced from the tunica adventitia are not established but it may be that pain is mediated by the plexus of beaded nerve fibres that lies between the adventitia and the media, for they are not unlike the pain terminals found elsewhere, and they are known to persist, at least in part, after ganglionectomy (Woollard *et al* 1940)

In order to determine the source and function of the different plexuses it is proposed to carry out dorsal root sections and sympathectomies, and to report in a future paper the results of these experiments

SUMMARY

1 The innervation of small blood vessels has been studied by intra-vital methylene blue staining, using a continuous drip technique

2 The nerves supplying the small arteries form plexuses (a) in the tunica adventitia, consisting of finely medullated and non-medullated fibres having mainly a direction parallel to the long axis of the vessel, (b) between the tunica adventitia and media, a meshwork of fine fibres, and (c) in the tunica media, a plexus of very fine beaded fibres

3 The arterioles are accompanied by a small number of nerve fibres, usually two or three, which in the larger arterioles may be placed on either side of the vessel with transverse inter-communications In the pre-capillaries only one fibre may be present, passing at intervals from one side of the vessel to the other

4 The capillaries are accompanied by very fine beaded fibres running close to the vessels, or, it may be, at some little distance from them

5 Medullated fibres are found ending among the fat cells that lie in close

proximity to the vessels Endings of an organized type are found in the tunica adventitia of the small arteries

6 It is suggested that the plexus lying immediately external to the tunica media may possibly subserve the sensation of pain and may also provide a mechanism for the production of active vaso-dilatation

7 Methylene blue, in concentrations greater than those required to stain nerve fibres specifically, frequently stains other tissues in addition

8 A classification of small blood vessels is suggested based on their innervation

My thanks are due to Prof W E Le Gros Clark for generously granting the facilities for work in his department I wish to thank also Dr G Weddell and his co-workers for their helpful criticisms and advice during the preparation of this paper To the technical staff of the Department of Anatomy at Oxford I am also most grateful

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EXPLANATION OF PLATES

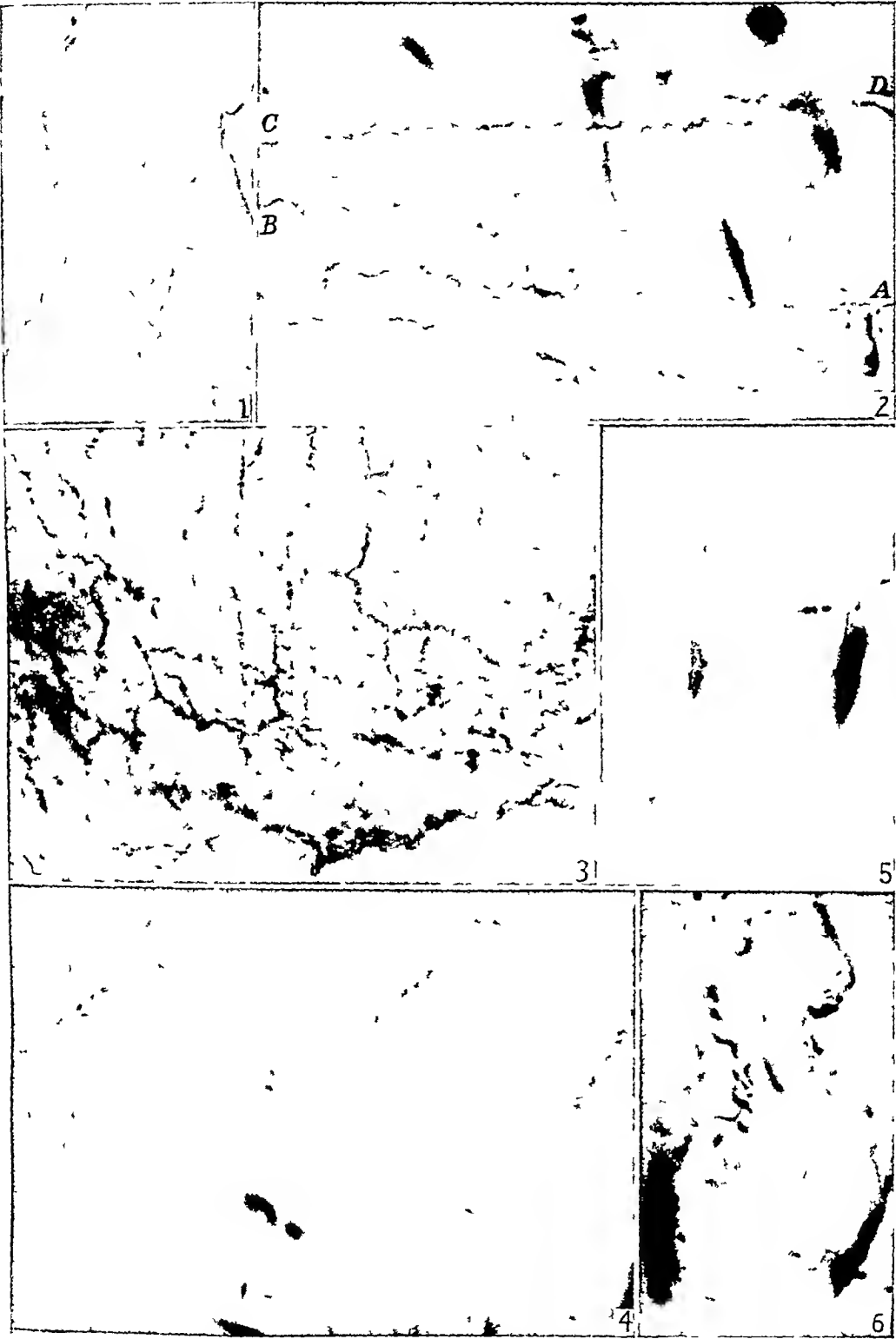
All the photomicrographs are of methylene blue preparations

PLATE 1

- Fig 1 Two small nerve fibre tracts and stained reticular connective tissue fibres $\times 500$
 Fig 2 Small artery from stomach of rabbit Adventitial plexus with longitudinal nerve fibres $\times 475$
 Fig 3 Plexus of varicose nerve fibres on the outer surface of the media of a small artery $\times 475$
 Fig 4 Plexus of fine beaded nerve fibres within the media of a small artery $\times 610$
 Fig 5 Beaded nerve fibre with knob like ending beside nucleus of smooth muscle cell $\times 1000$
 Fig 6 Organized ending in the adventitia of a small artery $\times 1200$

PLATE 2

- Fig 7 Medullated nerve fibre among fat cells beside a small vessel $\times 420$
 Fig 8 General pattern of innervation of an arteriole *A*, single nerve fibre $\times 110$
 Fig 9 Portion of the arteriole in Fig 8 under higher magnification $\times 450$
 Fig 10 Plexus of nerve fibres on the wall of an arteriole $\times 430$
 Fig 11 Arteriole with pre capillary branch $\times 510$
 Fig 12 Fine beaded nerve fibre on the wall of a capillary $\times 500$



This gave an opportunity of observing the various stages of healing in an injury to the skull

Groups B and C were both killed on the 4th day after operation. The animals of these groups were, in addition, given a daily intraperitoneal injection of a 1 % solution of trypan blue at the rate of 1 c.c. per 100 g. of body weight

All the tissues (pieces of parietal bone and of femur) were fixed immediately after death in ice-cold 80 % alcohol. Most of the pieces of skull were embedded in celloidin and undecalcified transverse sections $50\ \mu$ thick were cut from them. Some pieces of skull were decalcified in a vitamin C solution in the hope that paraffin sections could be obtained which would still contain active phosphatase, but without success. (Vitamin C if used in the dark, is a relatively good decalcifying agent. A 5 % solution has half the activity of a 5 % solution of trichloroacetic acid.) After the femora of the animals had been fixed for 24 hr. the repair tissue in the holes was scooped out and sectioned in the ordinary way. All sections were treated by Gomori's (1941) alkaline phosphatase technique

RESULTS

A *The healing process in the skulls of animals on a normal diet*

24 hr. Two main types of phosphatase positive cell were present in the blood clot filling the hole in the skull. The first type gave an intensely positive phosphatase reaction in the nucleus (Pl 1, fig 1), but the cytoplasm of some of the cells contained numerous dark granules, presumably representing sites of phosphatase activity, which made it impossible to distinguish the nucleus. These cells appeared to be polymorph leucocytes. The second type of cell gave a strong reaction in both nucleus and cytoplasm, and possessed elongated processes which also appeared to contain the enzyme (Pl 1, fig 2). In the central part of the hole other phosphatase positive cells resembling in general the second type appeared to join up to form capillaries.

Large numbers of the first type of cell were also present in both periosteum and endosteum, more were present in the former than in the latter. Not all of these cells in the periosteum stained in this way, but the number which gave a positive phosphatase reaction increased near the margins of the hole. About 4 mm. from the hole some eight out of every twenty polymorph-type cells in the periosteum were phosphatase positive (Pl 1, fig 3). At 3 mm. from the hole about fifteen out of twenty cells were positive. This was the same up to 0.5 mm. from the hole, but from there to the margin of the hole every cell was phosphatase positive. Some of the cells in the periosteum in which the whole nucleus had not blackened showed an intensely positive nucleolus. This increase in the number of phosphatase positive cells in the periosteum suggests that they migrated along it and that as they came closer to the area of injury they were under the influence of some chemical substance liberated from the injured tissue which caused them either to synthesize alkaline phosphatase or to absorb it.

3 days There were fewer of the polymorph leucocyte type of cells in the injured area or in the periosteum and endosteum. Numerous osteogenetic fibres were present in the hole and in the cellular layers of the periosteum near the hole. Many of these fibres gave a positive phosphatase reaction (Pl 1, fig 5). Most of the nuclei of the periosteal cells (cambial layer) gave a slight positive phosphatase reaction. More phosphatase positive capillaries were present.

1 week Two massive concentrations of phosphatase were present, one on either side of the repair tissue, about half-way between the outer and inner surfaces of the hole, and there were histological signs in these regions that formation of trabeculae was commencing (Pl 1, fig 6). There were practically no phosphatase positive cells in the periosteum at this stage.

2 weeks The numerous fibres in the repair tissue gave a positive phosphatase reaction and in certain areas—particularly where the concentration of phosphatase was heaviest, bony trabeculae could be seen forming. None of the positive polymorph-type cells, seen so plentifully in the 24 hr preparation, could be seen in the repair tissue or in the periosteum.

B *Animals on a scorbutic diet plus synthetic vitamin C*

The animals of this, and the succeeding, group were given trypan blue as described above. All four animals in this group were killed 4 days after operation. Numerous osteogenetic fibres were present in the repair tissue in most specimens. A large number of polymorph-type cells were present but only a small proportion of them had phosphatase positive nuclei. Numerous macrophages (detectable by their reaction to trypan blue, see Cappell, 1929) were present in the repair tissue, and in the endosteum and periosteum. Only a few were present in the cellular layer of the periosteum (Pl 1, fig 4, Pl 2, figs 7, 8). In one preparation a periosteal blood vessel was cut through near the margin of the hole and macrophages could be seen in the vessel. Macrophages could be seen outside the vessel also giving the impression that they were migrating out into the fibrous layer of the periosteum and then along this, guided by the fibres of that layer, towards the hole. Accumulations of macrophages could also be seen in some endosteal blood vessels and in some blood vessels in the bone itself (Pl 2, fig 9). So many were present in some of the vessels that near the injury the whole of the contents of the vessel appeared to stain a bright blue. It is noteworthy that the macrophages gave no phosphatase reaction.

C *Animals on a scorbutic diet*

All four animals in this group were killed 4 days after operation. In all specimens relatively few cells were present in the repair tissue filling the hole. There were some rounded cells containing coarse granules of trypan blue which gave no phosphatase reaction (these were macrophages). There were some cells with elongated processes some of which gave a slight phosphatase

reaction and some which gave no reaction at all. A few polymorph cells were present which gave a positive reaction. All preparations showed a few fine fibres and capillaries which were also positive. Near the edges of the hole where there was some necrosis and crumbling of bone, macrophages could be seen which contained masses of material that stained black with cobalt sulphide without prior incubation with enzyme substrate. This material was probably necrotic bone. It is of interest that the osteocytes in the uninjured bone do not take up trypan blue to any extent. Sections of material from the hole bored in the femur also showed a reduction in the number of cells present in the injured area in scorbutic animals. In bone marrow of both normal and scorbutic animals bone marrow cells gave a positive phosphatase reaction, and macrophages (stained with trypan blue but giving no phosphatase reaction), could be seen among them (Pl 2, fig 10).

DISCUSSION

This work shows that within 24 hr of injury to a skull bone there is an accumulation of cells, the nuclei of which give a positive phosphatase reaction, in the injured area and in the periosteum and endosteum near the region of injury. The cells are similar to those found by Fell & Danielli (1943) in injuries to the skin and called by them polymorphs. The polymorphs present in the injured area probably come from blood vessels in the periosteum and it is only when they approach the site of injury that they give a strong positive phosphatase reaction. The number of these cells migrating to the injured area in the skull bone is very greatly reduced in scorbutic animals, but the ability of the cells which do get to the injured area to give a positive phosphatase reaction does not appear to be affected. Vitamin C deficiency appears therefore to reduce the migratory powers of polymorphs, but does not completely inhibit the production of some substance from the injured area which apparently stimulates the production or absorption of phosphatase by the polymorph.

Osteogenetic cells and fibres give a positive phosphatase reaction when they first appear in an injured region. As the formation of trabeculae commences there is further and more intense accumulation of phosphatase in the cells and fibres at the site of formation of trabeculae.

There is thus a double cycle of phosphatase production by osteogenetic cells in the process of bone formation. The first when the osteogenetic fibres are produced, and the second when the fibres are collected together as trabeculae. A scorbutic diet not only reduces the number of polymorphs present in the injured area but also has an inhibiting effect on the production of fibres by the osteoblasts and reduces the number of capillary vessels formed.

It is of interest that Willmer (1942) found that normal fibroblasts, endothelial cells and macrophages in tissue culture could not be distinguished from one another by the phosphatase reaction, which is weak in all of them unless they are undergoing mitosis. In the present work, cells which appear to be forming capillaries (endothelial cells) and those forming fibres (osteogenetic

cells, which are a special type of fibroblast) gave similar phosphatase reactions. On the other hand, macrophages gave an almost completely negative reaction, although in some of them just the slightest blackening of the nuclear membrane could be seen. One must remember, however, that the conditions under which Willmer examined his cells (in tissue culture) were very different from the conditions under which the present author studied the same cells and the differences in staining reactions are almost certainly due to this fact.

The flood of macrophages which pours into the injured area confirms more indirect observations of the same phenomenon made by Macklin (1920). There is no evidence that these macrophages are formed by transformation of periosteal cells or modification of fibroblasts or other cells in the clot. Macrophages in the periosteum near the injury were concentrated in the fibrous layer, there were only a few in the cellular layer. They could be seen in periosteal blood vessels and in blood vessels in the bone. In the latter there was a very large concentration of them near the injury. While this is scarcely the place to enter into a discussion of the origin of macrophages, it might perhaps be said that the impression given by the preparations is that most, if not all, of the macrophages come to the injured area via the blood vessels. This agrees with the results of Eberth & Florey (1939).

There are fewer macrophages in the injured area in the vitamin C-deficient animals. This is in accord with previous observations that phagocytosis is inhibited in scurvy, e.g. Hunt (1941) found a delayed removal of catgut ligatures and of damaged muscle in vitamin C deficiency. Matzner (1938) has shown that macrophages in the lung in infections accumulate large stores of vitamin C within themselves so that the vitamin is presumably essential for their activity.

SUMMARY

This work has shown that within 24 hr. of injury to a bone, cells of which the nucleus gives a strong positive phosphatase reaction, and which are probably polymorphs, accumulate in the injured area and in the periosteum and endosteum near it.

Proximity to the injured area appears to accentuate the phosphatase reaction in these cells.

By 3 or 4 days after injury there are very few of these cells left of the injured area and they have almost completely disappeared after 1 week.

Numerous osteogenic and endothelial cells both of which give a positive phosphatase reaction are present in the injured area within 3 days of the injury.

Macrophages, which give no phosphatase reaction, are present in large numbers in the injured area at 4 days after injury.

The number of all varieties of cells present in the injured area after 4 days is greatly reduced by a scorbutic diet.

The healing process in the skull is very much slower than in the femur. A small hole bored in the femur of a guinea-pig on a normal diet is completely filled with bony trabeculae at the end of 1 week. In the skull only a few trabecular strands are present at the end of 2 weeks.

I am indebted to Roche Products for the supply of synthetic vitamin C used in these experiments.

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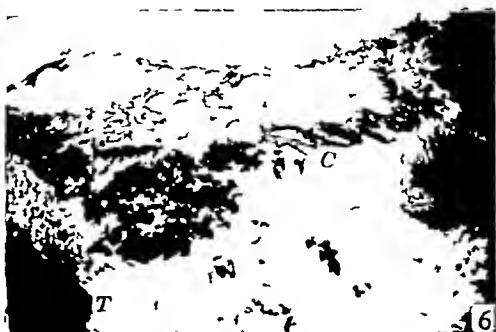
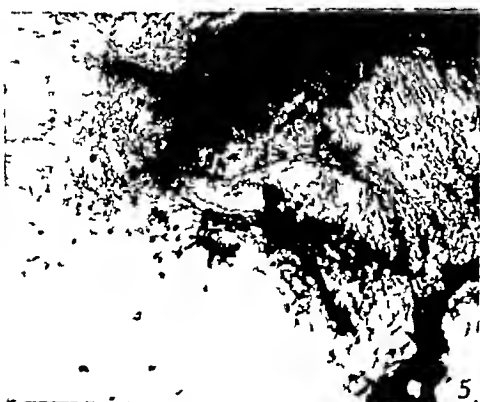
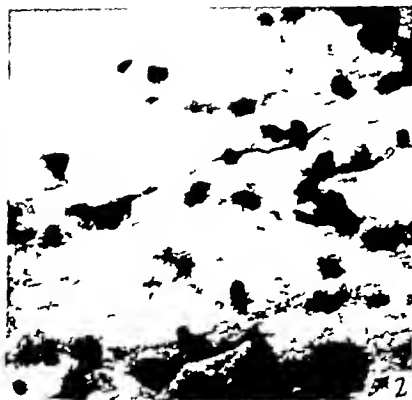
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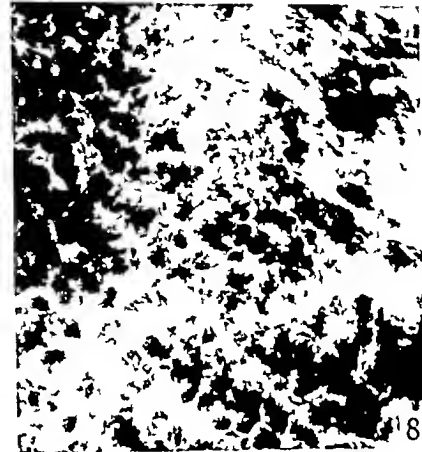
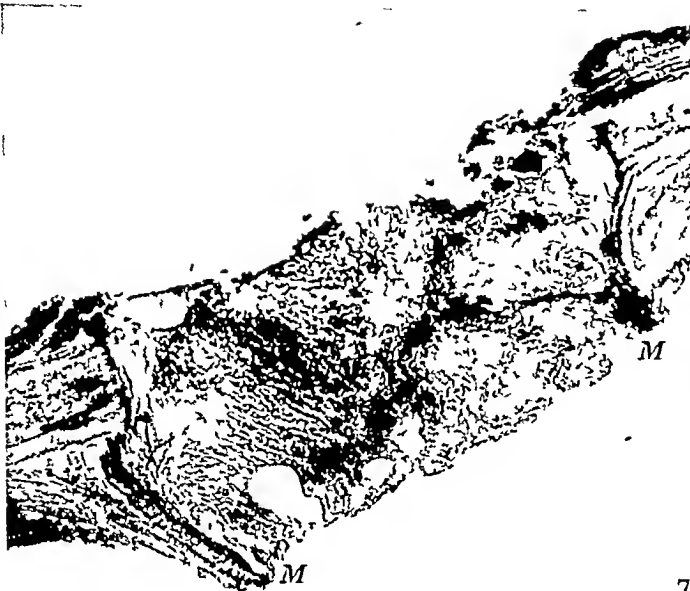
EXPLANATION OF PLATES

All photographs (except Pl. 2, fig. 10) are taken from undecalcified sections approximately 50μ thick. It was found impossible to cut satisfactory undecalcified sections of the guinea pig's skull at less than this thickness.

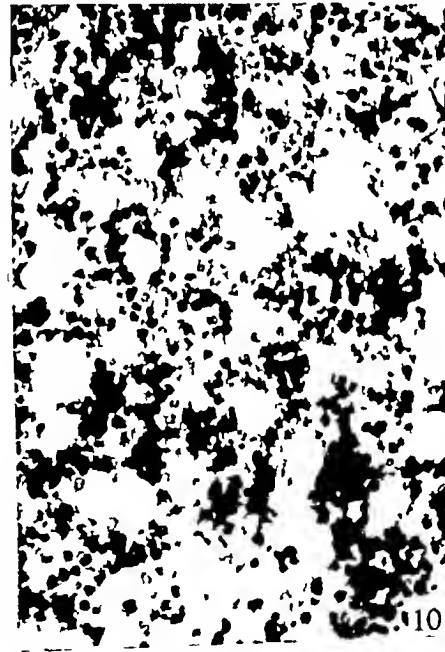
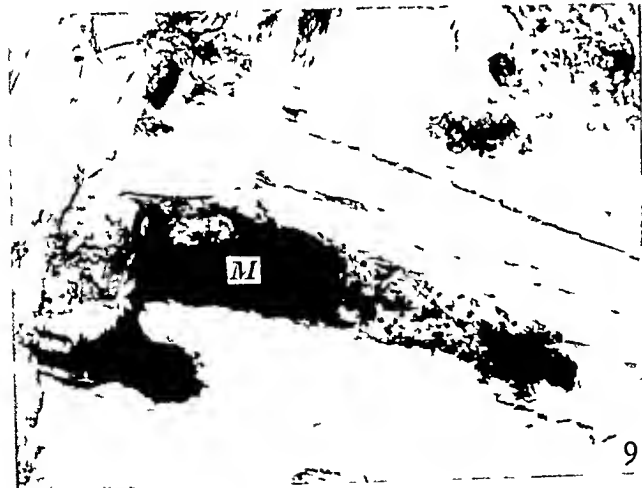
PLATE 1

- Fig. 1. Repair tissue in hole in skull 24 hr. after operation. Outer surface of bone uppermost. Numerous phosphatase positive cells (*P*) are present. A phosphatase positive capillary (*C*) can be seen in process of formation. The black mass at the top of the photograph is due to a fold in the section. (Phosphatase preparation. No counterstain.) $\times 65$.
- Fig. 2. Repair tissue from hole in skull 24 hr. after operation. Cells with nucleus and cytoplasm stained black can be seen. A blackened process (? osteogenetic fibre) can be seen extending some $40-50\mu$ from one cell. This suggests the production of a phosphatase laden fibre from a cell containing large amounts of the enzyme. Other cells with phosphatase mainly in the nucleus can also be seen in the photograph. $\times 650$.
- Fig. 3. Cellular layer of periosteum of skull about 4 mm. from hole. 24 hr. after operation. Large numbers of phosphatase positive cells (polymorphs) are present. $\times 120$.
- Fig. 4. Periosteum near hole in skull 4 days after operation, showing accumulation of macrophages in fibrous layer (*F*), but only occasional macrophages in cellular layer (*C*). (Intravital trypan blue.) $\times 120$.
- Fig. 5. Repair tissue in hole in skull 3 days after operation. Central black mass is formed of phosphatase positive fibres which can be seen more clearly on the right of the photograph. Only a few phosphatase positive cells are present. $\times 120$.





7



- Fig 6 Repair tissue in hole in skull 1 week after operation outer surface of bone uppermost Black material extending across near the top of the photograph is made up of necrosing chips of bone (*C*) The lateral black masses indicate the formation of bony trabeculae (*T*) That is the cells in these areas are commencing a second cycle of phosphatase production Isolated phosphatase positive cells can be seen at the margins of these black masses $\times 65$

PLATE 2

- Fig 7 Hole in skull 4 days after operation outer surface of bone uppermost Dark areas in repair tissue and at margins of hole (*M*) represent aggregations of macrophages The only stain used is intravital trypan blue $\times 65$
- Fig 8 Repair tissue from hole in skull 4 days after operation, showing masses of macrophages The granular character of the cells can be seen (Intravital trypan blue) $\times 250$
- Fig 9 Blood vessel near hole in skull 4 days after operation On the left macrophages are so numerous in the vessel as to appear as a black mass (*M*), but are not so numerous on the right and so individual cells can be seen (Intravital trypan blue) $\times 50$
- Fig 10 Repair tissue from hole in femur Phosphatase positive cells appear black. Macrophages which give a negative phosphatase reaction have trypan blue granules in the cytoplasm and appear grey in the figure (Intravital trypan blue and phosphatase technique) $\times 100$

CHANGES IN THE VOCAL FOLDS IN HUMMING LOW AND HIGH NOTES A RADIOGRAPHIC STUDY

By A G H MITCHINSON AND J M YOFFEY

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The object of the present study has been to investigate radiographically the movements of different parts of the larynx and related structures during alterations in the pitch of the voice

METHODS

Twenty subjects (ten male and ten female, ranging in age from 16 to 50) were X-rayed during the humming of (a) low notes, (b) high notes. Seventeen of the subjects were merely asked to hum at a pitch near the highest and lowest notes of their register. But three subjects who had done a fair amount of singing were given notes of known frequency by means of a tuning fork.

Humming was employed rather than singing, in order to eliminate movements of the jaw and structures immediately below it. But in so far as the changes in the vocal folds are concerned, the results are believed to be comparable. The head was immobilized as far as possible by one support placed against the occiput and another under the chin. The distance from tube to head was 5 ft., only lateral views were taken.

RESULTS

Change in length of vocal folds

A constant finding, as would be expected, was the increased length of the vocal folds in humming high notes (Pl 1, figs 1, 2, Pl 2, figs 3, 4). The maximum lengthening was 9 mm., minimum 2 mm., and the average was 5 mm. Cunningham (1943, p. 666) quotes figures from Moura (1879) to show that stretching may increase the length of the vocal folds by 4.5 mm. in the male and by 3 mm. in the female, without indicating how the stretching was effected, i.e. whether during life or passively in the cadaver. Negus (1929) quotes Cunningham's observation but gives no further information. Pressman (1942) studied the lengthening and shortening of the folds during life by direct laryngoscopic examination (see his Fig. 10, p. 374).

In the present series, the maximum elongation (9 mm.) occurred in a female. Furthermore, the practised singers (Table 1) showed greater elongation than the others. Thus in three practised singers, the average lengthening was 8 mm., ranging from 7 to 9 mm., while in the remainder the corresponding figures were 4.3 mm., ranging from 2 to 9 mm. (Pl 1, figs 1, 2, Pl 2, figs 3, 4).

The present study throws no light on the mechanism by which training could bring about this result, whether by increasing the mobility of the laryngeal cartilages, inducing hypertrophy of the laryngeal muscles, or both, but about the result itself there appears to be no doubt. Comparison of Pl 2, figs 3 and 4 shows that in this particular instance the noteworthy feature is the greater shortening of the folds in the low note.

Table 1 *Changes in vocal fold length in practised singers*

	Low note		High note		
	Frequency of note	Length of fold (mm)	Frequency of note	Length of fold (mm)	Lengthening of fold (mm.)
Miss B	144	8	852	17	+9
Mr S	106	10	516	17	+7
Mr W	96	16	341	24	+8

It has not been found possible to obtain for examination fully trained singers, to ascertain whether their vocal folds are capable of still greater elongation. Conceivably, also, radiological examination before embarking on a course of voice training might provide an objective criterion for assessing the probability of a successful result.

The vestibular folds are elongated at the same time as the vocal folds, and the sinus frequently becomes narrower (Pl 1, figs 1, 2), on an average by 1 mm and in one instance by 2 mm. Narrowing of the sinus has previously been noted by Curry (1937).

Rotation of vocal folds

In changing from low to high notes the vocal folds frequently (thirteen cases out of twenty) undergo rotation downwards and forwards (average 7°, maximum 20°, minimum 0°). This is due to rotation between the cricoid and thyroid cartilages, and appears to depend mainly on the action of the cricothyroid muscles. The action of these muscles has long been a matter of controversy in which some of the great figures in anatomy have participated (for a brief historical review see Tschuassny, 1944). Fabricius ab Aquapendente wrote 'at Ego de usu horum musculorum anceps hucusque fui' (quoted by Jurasz, 1901). Winslow (1732) expressed the view that 'Les Crico-Thyroidiens sont disposés d'une façon qu'il est difficile de déterminer leur usage. Ils peuvent ou faire reculer le Cricoïde, ou faire avancer le Thyroïde, et cela plus obliquement de bas en haut et de devant en arrière'. The main question has been whether the cricoid cartilage remains fixed, while the thyroid cartilage rotates downwards and forwards, or vice versa. Negus (1929) advocates the former view, and attributes the fixation of the cricoid cartilage to the cricopharyngeus muscle. If this be so, then the anterior end of the vocal fold should move forwards as the fold elongates, and the distance between it and the vertebral column should increase. However, the same result could follow if the cricoid were the movable cartilage, and the larynx as a whole were displaced forwards.

In the normal larynx the cartilages are so radio-translucent that it is difficult to observe finer degrees of movement directly. However, in one of our subjects in whom the thyroid cartilage was calcified, rotation of the thyroid was clearly seen and amounted to 6° . This is very close to the average figure for rotation of the vocal folds, namely 7° , noted in the preceding paragraph, although of course one must not attach too much importance to a single observation.

The larynx as a whole undoubtedly moves upwards in humming high notes (Pl 1, figs 1, 2, Pl 2, figs 3, 4), as indicated by the corresponding upward displacement of the vocal folds (average distance 1 vertebra, maximum 2 vertebrae, minimum $\frac{1}{2}$ a vertebra). This is associated with elevation, though to a less extent, of the hyoid bone, which also undergoes rotation (Pl 1, figs 1, 2, Pl 2, figs 3, 4). The upward movement of the larynx makes it difficult to be sure of the extent of forward displacement. Cine-radiography would probably give a clear-cut answer to the problem, but unfortunately was not available. Some forward displacement seems definitely to occur, for if one measures the distance between the anterior extremity of the vocal fold and the vertebral column, it increases in the high notes by a maximum of 1.3 cm, whereas the maximum elongation of the vocal fold was only 9 mm. Furthermore, increase in the antero-posterior thickness of the prevertebral soft tissues can frequently be seen (Pl 1, figs 1, 2, Pl 2, figs 3, 4).

Apart from the possible restraining influence of the crico-pharyngeus on movement of the cricoid cartilage, the ary-epiglottic muscles may also exert an effect. In high notes the hyoid bone is drawn forwards an average of 1 cm, and with it the epiglottis. The pull of the ary-epiglottic folds would indirectly prevent the backward rotation of the cricoid. The position of the arytenoids is indicated by the shadows cast by the soft tissues surrounding them, in humming high notes there is no suggestion of the arytenoid movement which might be expected to accompany rotation of the cricoid. Besides the arytenoids—or rather the tissue mass around them—the line of the ary-epiglottic folds is also evident in a good X-ray.

It is generally assumed that the only movement possible between the thyroid and cricoid cartilage is one of rotation around an axis passing transversely through the two crico-thyroid articulations. But this may not be the case. In dissecting-room specimens where the muscles and ligaments have been hardened by preservative, movement of any kind between the cricoid and thyroid cartilages is difficult to demonstrate. But in a fresh and unfixated larynx it becomes evident that, besides rotation, an appreciable antero-posterior gliding movement of the thyroid on the cricoid may take place, and this no doubt is effected by the more horizontally placed fibres of the crico-thyroid muscle.

The supra-glottic space

The shape and size of the supra-glottic air space must be varied to alter the resonating frequency of the air column, so that it corresponds with, and is adjusted to, the vibratory frequency of the vocal folds. Thus in the high notes

the supra-glottic space is shortened, just as in an organ the higher the note the shorter the organ pipe employed. The supra-hyoid muscles, as well as the stylo-pharyngeus and palato-pharyngeus, are mainly responsible for this shortening of the supra-glottic space. In the case of the hyoid bone the chief action seems to be exerted anteriorly, since the bone is not only elevated but also rotated backwards.

Furthermore, in the high note the larynx is approximated to the hyoid bone, and what we have termed the hyo-vocal gap (between the hyoid bone and the vocal cords) is diminished. This is presumably due to the contraction of the thyro-hyoid muscles, and has the effect of shortening a segment of the supra-glottic cavity, and further assisting in reducing the height of the resonating column of air.

The supra-glottic cavity is not only shortened, but also widened antero-posteriorly through the forward displacement of the hyoid bone (Pl. 1, figs 1, 2).

Finally, it may be noted that the elevation of the larynx is associated with a characteristic elongation of the trachea, and a straightening of its anterior border. In humming low notes, when the larynx is depressed, the anterior margin of the trachea as seen in a lateral radiograph shows a characteristic forward convexity. In the high note, with tracheal extension, this disappears.

SUMMARY

Lateral radiographs were taken of the larynx of twenty subjects, humming low and high notes. In changing from a low to a high note the vocal folds usually become elongated, and rotated. The larynx and hyoid bone are raised and the supra-glottic space shortened.

In three subjects with considerable practice in singing, the elongation of the vocal folds was greater than in the remaining seventeen subjects.

We are indebted to Prof. J. C. Brash for a photostat copy of Moura's (1879) paper, and to Mr Joseph Dann for assistance with the radiographs.

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EXPLANATION OF PLATES

PLATE 1

Figs 1, 2 X rays of the larynx of an 18 year old male subject humming a low note (fig 1) and a high note (fig 2) Note the elongation and rotation of the vocal folds, the narrowing of the sinuses, and the forward and upward displacement of the larynx Note also the movement of the hyoid bone In fig 1 the line of the ary epiglottic fold can be clearly seen

PLATE 2

Figs 3, 4 X rays of the larynx of a practised singer, female, age 21, during the humming of a low note (fig 3, frequency 144) and high note (fig 4, frequency 852) Note the shortness of the vocal folds in fig 3 Note also the tracheal elongation with straightening of the anterior border, the elevation and rotation of the hyoid bone, and the increased thickness of the provertebral soft tissues in fig 4



Fig 2



Fig 1



Fig. 4



Fig. 3

DENDRITIC CELLS

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INTRODUCTION

It has been known for many years that in pigmented mammalian epidermis and in the matrices of the hairs, branching cells containing melanin granules (variously described as melanoblasts, melanophores, dendritic cells, stellate cells, chromatophores, *cellules amboceprices*, etc) can sometimes be distinguished in addition to the ordinary, rounded, epidermal cells. None of the several theories which have been put forward at various times concerning the origin and functional significance of these branching cells has, however, so far attained general acceptance (see reviews by Hoepke, 1927, Percival & Stewart, 1930, Meirowsky, 1940, Laterjet, 1938), though most authorities would agree with Meirowsky's (1940) statement that 'the central problem in pigment research is the origin and significance of the dendritic cells'. The 'cell of Langerhans', a branched cellular component of the epidermis originally described by Langerhans (1868) in normal human skin that had been impregnated with gold chloride, although regarded by some authorities (Woollard, 1935, Cowdry, 1938, Carleton, 1938) as identical with the branching, pigmented cells for which numerous synonyms have already been given above, will be considered separately here, since Langerhans specifically denied that this element had anything to do with pigmentation and regarded it as a nervous element—a view subsequently supported by Bloch (1929). This view implies, of course, that these cells are distributed generally throughout the epidermis and are not restricted to the pigmented regions.

It is probably true to say that in spite of all that has been written on dendritic cells the great majority of histologists regard the non-pigmented epidermis (and in many cases the pigmented epidermis also) as a homogeneous tissue in the composition of which structure only one type of cell participates.

In the present study a re-investigation of the anatomical basis of pigmentation of the mammalian epidermis has been undertaken (Part I of this paper) and the results of this investigation have been applied to an analysis of the striking system of branching elements observed in non-pigmented epidermis (Part II). Following Becker (1927) the term 'dendritic cell' will be used as a purely morphological description in preference to any of the other synonyms, since it begs no question about the origin and functional significance of the cell.

PART I THE DENDRITIC CELLS IN PIGMENTED EPIDERMIS

MATERIAL AND METHODS

In this study black and white guinea-pigs have been used throughout. These animals were particularly suitable since in the areas that bear black hairs the superficial epidermis itself is deeply pigmented while in the white hair-bearing

regions the skin is non-pigmented. In other laboratory mammals, by contrast the superficial epidermis of the skin that bears pigmented hairs is not pigmented. This is so in the case of the body skin of the rabbit, but in the skin of the dorsum of the ear in which the hair density is relatively low, some epidermal pigmentation does occur.

The epidermis of the ear of the guinea-pig, although differing in no essential details from that of the body skin with respect to pigmentation, proved more favourable for this investigation on account of its relatively hairless nature and also because it was much thicker than the body epidermis.

Fixation and staining Formol-mercuric chloride, prepared by mixing saturated mercuric chloride solution, 40 % formaldehyde, and distilled water in the ratio of 50:15:35 parts by volume, was adopted as a standard fixative. Paraffin sections were cut at 8–10 μ and lightly stained with Ehrlich's haematoxylin and eosin. To study the distribution of melanin sections were either mounted unstained or were lightly stained with Orange G.

Studies with living cells These were carried out upon sheets of 'pure', pigmented epidermis—i.e. epidermis which had been completely freed from dermal material (Pl. 1, figs. 1, 2) and upon emulsions consisting of isolated cells and undissociated clumps of cells. These were prepared by the tryptic digestion of thin skin slices and the subsequent separation of the epidermis from its underlying collagen pad as described by Medawar (1941). This method is referred to hereafter as 'skin splitting'. It will be shown in another publication (Billingham & Medawar, 1948) that the epidermal sheet remains alive after this treatment.

The Dopa reaction When freshly excised tissue is incubated at 37° C. with a 1:1000 solution in phosphate buffer at pH 7.4 of *l*-dihydroxyphenylalanine ('Dopa'), a likely precursor of melanin (Raper, 1927), certain cells, including those known to be capable of forming melanin, bring about the rapid intracellular oxidation of this substance thereby becoming blackened by the resultant Dopa-melanin. These cells are said to be 'Dopa positive'. The standard procedure of Laidlaw & Blackberg (1932) was carried out upon frozen sections of full thickness skin and upon sheets of pure epithelium. Counter-staining was found to be unnecessary, but it was found advisable to fix the fresh tissue, e.g. in 'formal calcium' (Baker, 1944) for 2 hr. before cutting in order to harden it.

Studies on hyperplastic skin These were carried out upon pigmented ear skin which had been rendered hyperplastic by grafting it to a recipient area on the animal's own thorax (Medawar, 1944). About 12 days after transplantation (the most favourable time) thin shavings of the thickness of Thiersch grafts were cut from the graft and 'split'. Both the resultant sheet of epidermis and the collagen pad were then fixed, dehydrated, and cleared and mounted unstained.

OBSERVATIONS

General histology of the epidermis The pigmented auricular epidermis consists of about six layers of cells and is penetrated by well-defined dermal papillae. The melanin granules are located mainly in the cells of the basal layer in contact with the corium. These cells are of cylindrical shape with their long axes disposed in the direction normal to the plane of the integument. The granules are generally heaped up over the poles, usually the superficial poles, of the nuclei. Other granules may be distributed more evenly throughout the cytoplasm of these cells. In the more superficial layers in which the cell bodies are more rounded, the polar distribution gives way to a more even one. One gets the impression that as the cells move towards the stratum granulosum the quantity of melanin in each cell decreases. This is probably illusory as the cells become more superficial the volume of the cytoplasm in relation to that of the nucleus increases and the original complement of melanin granules becomes diluted.

Melanin granules are to be seen also in the squamous cells of the stratum corneum.

In paraffin sections of pigmented skin dendritic cells can only rarely be distinguished. This may be due to the fact that they are obscured by the rather dense pigmentation of the basal layer. A few dendrites are occasionally visible by virtue of their pigment content, or a large, heavily pigmented cell body may be seen disposed horizontally and lying so deep in the basal layer as to touch the dermis.

In transverse sections of pigmented epidermis which had been prepared by 'splitting' the dendritic cells and some of their processes stand out more clearly (Pl 1, figs 2-4). This is probably attributable to the fact that as long as the epidermis is anchored down to the dermis the individual cells are under slight lateral compression, but when the basal layer is freed from its substratum all the cells, particularly the basal, are loosened, thus permitting the dendritic cells, and the processes from them which run in the intercellular spaces, to become apparent. It is highly probable that these intercellular spaces in the deeper strata of the epidermis are all permeated by the dendrites of these branching cells. The so-called 'intercellular substance', so far as the epidermis is concerned, may be none other than the protoplasmic extensions of dendritic cells.

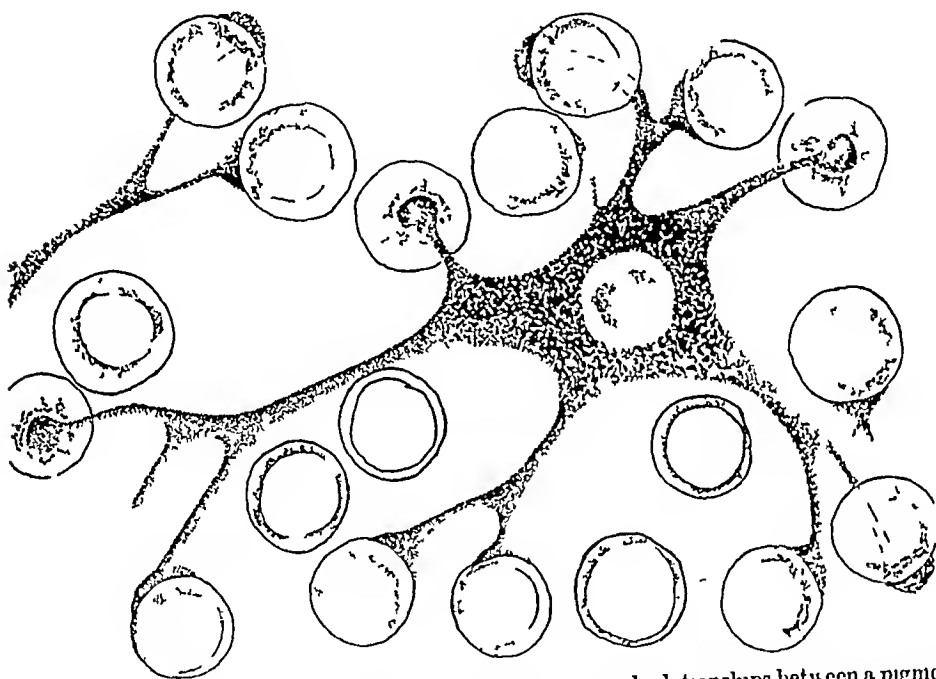
The epidermis of the body rarely exceeds in thickness two layers of cells including the basal layer, and dendritic cells or their processes are rarely seen in sections unless the skin has previously been rendered hyperplastic. The dermal papillae, which are feebly developed, are broad and shallow.

In both types of pigmented skin small, round, shield-shaped conglomerations of melanin granules may occasionally be seen lying in the 'intercellular spaces' at about the level of the basal layer.

Studies with living cells The isolated cells which can be observed in suspensions prepared from pure, pigmented epidermis can be divided into three groups

according to size and the relative volume of the cell occupied by the nucleus. Most of the cells in each group contain melanin granules.

Cells of the first group are those of the basal layer, and are the smallest. They are roughly spherical in shape and have very little cytoplasm round the nucleus. Pigment, when present, occupies a polar-capping position (Pl 1, fig 5, and Text-fig 1). The melanin granules associated with these cells do not, however, always form a cap *within* the cell membrane, for careful study with the oil immersion lens reveals that there may be a cap-shaped aggregate of granules either within the cell or closely applied to, but definitely *outside*, the cell membrane.



Text fig 1 Illustrating rather diagrammatically the anatomical relationships between a pigmented dendritic cell and its ordinary basal layer cell neighbours. Several cells have been figured in which the end caps have broken away from their dendrites. The pigment granules, which are produced within the cytoplasm of the dendritic cell, pass across the boundaries of the ordinary epidermal cells via the end caps and, in the majority of cells of the lower epidermal strata, remain in a localized 'polar capping' position with respect to the nucleus. This distribution normally gives way to a more even one as the cells reach the more superficial layer of the epidermis.

These extracellular melanin caps nearly always appear incomplete, so giving the impression that they have broken away from some other structure of which they were once a part.

It seems fairly certain that the shield-shaped conglomerations of pigment mentioned above are identifiable with melanin caps of this nature that have broken away from the cells that 'wore' them.

The second group is composed of cells which are slightly larger than those of the basal layer and which possess relatively more cytoplasm. By dark ground

illumination 'prickle' remnants may frequently be seen projecting from their membranes. These cells lie between the basal layer and the stratum granulosum. Pigment, when present, is distributed evenly throughout their cytoplasm and the 'caps' are very rarely seen.

Cells of the third group are rarely present in suspensions prepared by *lightly* scraping the undersides of pure epidermal sheets. They are larger in surface area than those of the other groups, being normally flattened, and their cytoplasm is packed with semi-transparent globules of eleidin. They usually contain scattered melanin granules.

Intimately bound up with the undissociated clumps of basal layer cells present in these suspensions, there can be observed dark bodies which are more irregular in shape and of slightly larger size than the basal layer cells. These elements are so completely filled with melanin granules, or at least the periphery of their cytoplasm is so completely lined with pigment granules, that they appear homogeneously black and no internal structure can be distinguished. These are the cell bodies ('perikarya') of the dendritic cells, and from them stumpy processes arise and travel outwards to pass between the surrounding basal layer cells.

If the amount of fluid between slide and coverslip has been correctly adjusted these clumps of cells gradually flatten out to form a single but still united layer of cells which is highly suitable for the detailed study of dendritic cells. Under such conditions the cell-bodies of dendritic cells appear elongated, rarely spherical, and generally have a maximum diameter of $14-16\mu$ and a minimum diameter of $8-12\mu$. The maximum diameters observed were $19 \times 18\mu$, but this degree of elongation is rare. (Ordinary basal layer cells vary in diameter between 11 and 14μ .) From the bodies of these cells a number of primary dendrites arise: they vary in number from 2-7, 5 and 6 being modal. These primary dendrites branch peripherally, mainly by dichotomy, and as they travel outwards there is a progressive diminution in their calibre. Proximally their width may be very variable. In the dendritic cells of ear skin it may be as much as 2.5μ at their point of origin from the cell body. Dendritic cells of body skin have longer processes—their maximum length may be as much as 100μ —but the primary dendrites at their point of origin are much smaller, having a diameter of between 1 and 1.5μ .

The finer 'twigs' of these branching dendrites can nearly always be seen to terminate in the vicinity of basal-layer cells in the form of a swelling which is button or cap like in shape (Pl. 1, fig. 8). This terminal cap, like the finest ramification of the dendrite itself—conspicuous entirely on account of its melanin granule content—appears to be intimately applied to the membrane of the basal cell (Text-fig. 1, Pl. 1 fig. 8) and differs in no respect from the polar caps described above in relation to the isolated basal layer cells.

Fusion between processes originating from the same dendritic cell has not been observed, but instances of fusion between processes originating from *different* dendritic cells can be found by careful examination of these flattened

cell suspensions (Pl 1, fig 9) Moreover, it may be established that an end-button from one dendritic cell may be applied to the body of another

The melanin granules contained within dendritic cells are approximately constant in size and are in all respects similar to those seen elsewhere within the epidermis

At the centre of the body of a dendritic cell a pellucid sphere, presumably the nucleus, can usually be distinguished (Pl 1, figs 6-8) When the pellucid sphere of a suitably flattened dendritic cell body is examined under an oil immersion objective by phase contrast microscopy a faint indication of the existence of a nuclear membrane and nucleolus is obtained If a drop of acidified methyl green is then allowed to run in under the edge of the coverslip and the same dendritic cells examined *without* the use of phase contrast, the nuclear membrane and contained nucleolus can be identified with certainty

The Dopa reaction In sections Dopa-positive cells were found only in pigmented skin (see p 104, footnote) They were invariably dendritic in form, their bodies, slightly larger than the basal layer cells, were usually disposed horizontally and so deep in the basal layer as frequently to touch the dermis From their bodies, which were deeply blackened by Dopa melanin, equally blackened processes arose laterally and apically, and branched among cells of their own and superficial layers In control sections which had not been treated with Dopa these dendritic elements could only very rarely and with difficulty be identified and then only by virtue of their natural melanin content There could be no doubt that the elements made clearly visible in pigmented skin by means of the Dopa reaction were identical with the dendritic cells from living material described above

Since dendritic cells branch principally in the plane of the epidermis transverse sections are obviously unfavourable material for studies of their numerical incidence and the extent of the 'zones of influence' of their branches This can be carried out best on whole mounts of pure pigmented epidermis mounted upside down In Dopa and non-Dopa material the picture was essentially the same save that in the Dopa material there was more contrast between the dendrites and other cells and the final terminations of the dendrites were more readily visible (Pl 2, fig 10) Under the high power the terminal buttons are seen to be strongly Dopa-positive, the basal layer cells with which they are in contact are however scarcely visible, since they are Dopa-negative and such natural melanin as they possess lies normally very close to the cap-like endings of the dendrites

A comparison of the dendritic cell content of black body skin and of pigmented ear skin, based on the study of both living and Dopa-treated material, reveals that in both there is but a single layer of dendritic cells, interspersed among the basal layer cells In ear epidermis the concentration of dendritic cells is much higher and they are found mainly in the epidermal re-entrants between the dermal papillae (Pl 1, fig 1) while in body epidermis they are most highly concentrated at the bases of its broad and shallow epidermal ridges

Studies on hyperplastic skin In the week or two that follow the transplantation of a skin graft the mitotic activity of its epidermal cells is greatly increased. If dendritic cells do in fact enjoy a cell lineage of their own, i.e. if they breed true to type instead of originating as induced modifications of basal layer cells, it seemed reasonable to hope that specific division stages would be found in hyperplastic skin.

Examination of pure hyperplastic epidermis (from 'split skin') revealed that its melanin content was sub-normal. The majority of the basal layer cells were completely devoid of melanin, and the dendritic cells, although no less numerous than in normal skin, contained only about half the expected quantity of pigment granules*. Moreover, many of them were undergoing division. From the various stages illustrated (Pl 2, figs 11-14) it is clear that in crude outline the mode of division resembles that of an amoeboid cell in the sense that the dividing dendritic cell withdraws its processes and forms a pigmented, rounded sphere about 12μ in diameter (measured from fixed material) (Pl 2, fig 11). This becomes dumb-bell-shaped by constriction in an equatorial plane (Pl 2, fig 12). At this stage daughter nuclei become visible. The two lobes of the dumb-bell draw apart and thick, blunt processes appear (Pl 2, fig 13). Even after the bodies of the daughter cells have separated, and each has developed a fairly complex system of dendrites, daughter dendritic cells can often be seen linked together by a common process (Pl 2, fig 14).

PART II DENDRITIC CELLS IN NON-PIGMENTED EPIDERMIS

MATERIAL AND METHODS

In this study the non-pigmented skin of spotted guinea-pigs, of rabbits and of man has been used. In the case of the rodents the epidermis of the ear proved more favourable for reasons which have already been given. The human material consisted chiefly of trimmings from Thiersch grafts made available through the kindness of Prof T. Pomfret Kilner (i.e. very thin grafts comprising epidermis and the superficial part only of the dermis), which had been cut from the forearm.

Gold impregnation The technique adopted was essentially that described by Garms (1930) as a reliable method for demonstrating the nerve endings in muscle. The human Thiersch graft material, or in the case of the rodents pieces of skin cut as thin as possible (in either case the material must be fresh) was placed in a mixture of one part pure formic acid and three parts filtered lemon juice and left in the dark for 10 min. or until it had cleared. The material was then removed from the liquid and lightly blotted on a filter-paper. It was then transferred to a 1% aqueous solution of gold chloride and returned to the dark.

* These observations are in conformity with the fact that a graft of pigmented skin, studied microscopically, seems to lose its colour temporarily following transplantation, taking on a pinkish colour at the end of the first week and gradually darkening thereafter until its former level of pigmentation is regained after about 3 weeks (see Billingham & Medawar, 1948).

for a further period of 10 min after which it was again blotted and subsequently placed in a 25 % aqueous solution of formic acid and left in total darkness for 24 hr. Finally the tissue was again blotted and placed in pure glycerine and left until it had cleared. The epidermis with most of its deeper strata intact was then fairly easily stripped from the dermis with the aid of fine forceps. It was then mounted in glycerine, basal layer uppermost, together with any epidermal material which could be obtained by lightly scraping the upper (i.e. surface which was applied to the epidermis) surface of the stripped collagen pad. This latter part of the technique is similar, manipulatively, to that previously described for skin splitting. When the shavings of skin were very thin they were teased and mounted directly after impregnation. (This technique, although probably reliable for the purpose for which Garms described it, has not invariably been successful as a method of staining white dendritic cells.)

Methylene blue staining Thin shavings of living skin were used with as little of the dermis left adherent as possible. These shavings were mounted on slides, dermal side uppermost, in a 0.02 % solution of methylene blue in Ringer's solution (B D H methylene blue, batch number 519420, has given satisfactory results). After several minutes a few drops of a 0.2 % solution of the dye in Ringer were drawn under the coverslip and the preparation was examined periodically with the microscope. After 1-2 hr it was observed that the tissue as a whole had remained relatively unstained but in the peripheral region of the epidermal sheet certain elements, which appeared to have a branching form, had taken up the stain selectively. At this stage the tissue was dehydrated, cleared and mounted in Canada balsam after treatment with saturated ammonium picrate solution and Bethe's fluid as described by Carleton (1938).

Azan staining Full thickness non-pigmented skin was fixed in formal-mercuric-chloride, dehydrated and cleared by passage through the alcohols, cedarwood oil and ligroin. After paraffin embedding, sections were cut at 10μ and at 15μ and stained by Heidenham's 'Azan' method as described by Pantin (1946).

OBSERVATIONS

Gold impregnation The superficial layers of the epidermis, comprising the stratum granulosum and stratum corneum, were found to be stained a deep purplish black by the presence of fairly coarse particles of reduced gold. The deeper layers of cells, including the basal layer, were found to vary in colour from pink to deep red. Only the nuclei of the cells in this case were stained and whatever the nature of the colouring matter, it was not visibly particulate. The actual boundaries of these cells could be made out only with difficulty. The dermis rarely took on more than a deep pink colour. In successful preparations, at about the level of the basal cells of the epidermis, dark reddish purple bodies of slightly fusiform but irregular shape could be seen (Pl 2, fig 15) which were, on the average, slightly larger than their ordinary epidermal neighbours. These bodies were made visible by the presence of very

fine granules of metallic gold within their substance and only with difficulty could a nuclear outline be distinguished. This latter fact is not very surprising since, in effect, one was trying to distinguish a pale reddish body surrounded by a mass of dark coloured particles.

From these bodies a variable number of branches or dendrites arose (Pl. 2, figs 16, 17). On an average there were about five of these but any number between two and ten have been observed. When there were but few branches, their initial diameter tended to be greater than in the case of the multi-branched elements. The mode of branching of these dendrites differed in no respect from that of the pigmented dendritic cells which have already been described. The processes likewise occupied the intercellular spaces between the basal and more superficial cells and some exceeded 70μ in length although the majority varied between 30 and 40μ . The finer twigs of the branches seemed to be rendered rather brittle by gold impregnation and were often found to have broken away from the parent cell. In spite of this, it could be established beyond doubt that they terminated as small, button-shaped bodies in close proximity to the boundaries of the basal-layer cells. These processes travelled both horizontally and towards the surface but not deeply. By careful searching, cases could be found in which end-buttons from these gold-impregnated dendritic bodies ended, not on an ordinary neighbouring epidermal cell but on the perikaryon of an element of its own type. Further, in several instances, two of these branched bodies were observed to be united by a common dendrite.

From the above description of the branching elements which can be shown to exist in non-pigmented epidermis by gold impregnation it will be seen that they closely resembled pigmented dendritic cells with respect to their size, shape, position, number, mode of branching, number and length of branches, the disposition and mode of termination of the branches or dendrites, the relations which may exist between two such dendritic cells and finally their nucleate nature. Merely on superficial inspection the branched elements of non-pigmented skin qualify to be called 'white' dendritic cells and to describe them more fully would result only in a repetition of what has already been said about pigmented dendritic cells.

In spotted guinea-pigs the non-pigmented epidermis of the sole of the foot (hairless and heavily cornified as in man), of body skin and of ear skin have all been found to contain white dendritic cells.

Apart from the fact that white dendritic cell bodies, as revealed by metallic impregnation, are of slightly more irregular shape and that their processes are, on an average, of finer diameter than in the case of the pigmented dendritic cells there are no other purely morphological grounds upon which pigmented and non-pigmented dendritic cells can be separated. As a critical test of the possibility that the very slight differences in form might be fixation artefacts, the pigmented skin from a guinea-pig's ear was treated by the full gold chloride impregnation method. In the resulting preparations there was no difficulty in distinguishing between granules of melanin and reduced gold chloride, because

the former appear brownish in transmitted light. In very heavily pigmented dendritic cells the body remained rounded in shape, probably on account of the mechanical resistance to fixation-induced shrinkage offered by the densely packed melanin granules. On the other hand, those dendritic cells which were more lightly pigmented assumed a form similar in all respects to that of the white dendritic cells, and their finer twigs were revealed with even greater clarity than in vital preparations of pigmented dendritic cells.

The white dendritic cells of human skin Non-pigmented* human skin from the face, the arms and the abdomen was examined. White dendritic cells were found in the epidermis in each case and were similar in all respects to those which have been described in the case of the non-pigmented skin of the guinea-pig (cf Pl 2, figs 16, 17).

Methylene blue staining was applied to skin from the human forearm, from the dorsum of the ear of albino and black-and-white rabbits and to the white ear skin of spotted guinea-pigs.

In each case elongated and irregularly shaped bodies could be seen in the epidermis at about the level of the very feebly stained ordinary basal layer cells. They were slightly larger than the neighbouring epidermal cells and a variable number of branches arose from them (Pl 2, figs 18, 19). Cell bodies and branches were rendered visible by the presence of numerous, brightly stained blue granules within their substance. It is unfortunate that no cytological detail could be distinguished within these branching bodies. With respect to their size, shape, numerical incidence, location and number of branches to which they gave rise, they were strikingly similar to the white dendritic cells revealed in control material which had been treated with gold chloride. In the methylene blue stained material, however, the cytoplasmic bridges, which are said to traverse the intercellular spaces and unite the deeper epidermal cells, took up the stain strongly (Pl 2, fig 19), particularly at their nodes, which made it impossible to trace the branches very far from their points of origin or to investigate their mode of termination. On many occasions it was found that the finer ramifications of the cutaneous nerves had stained particularly well but in no case was there any indication that these were related to the branching cells (Woollard, 1935).

Azan staining In sections of full thickness, of non-pigmented human skin stained by this method and examined with the one-sixth and one-twelfth objectives it was observed that certain cells in the basal layer of the epidermis were conspicuous by their larger size and by often being slightly elongated in the horizontal plane. A much thicker layer of cytoplasm surrounded their nuclei than in the case of the neighbouring epidermal cells (Pl 2, fig 22), and although clearly defined 'prickles' were present around the boundaries of most of the latter (Pl 2, fig 22), in no instance were they found around the former.

* Through the particular kindness of Dr H. M. Hanschell I have now been able to study the pigmented dendritic cells in negro skin. These are essentially similar to those found in pigmented guinea pig skin.

(Pl 2, figs 20, 22) In fact the lack of prickles constituted the most obvious diagnostic character of these large cells. It could be established that fairly large protoplasmic branches arose from the lateral and distal boundaries of these cells, and these, by careful focusing, could be followed for a short distance as they entered the adjacent intercellular spaces between the ordinary epidermal cells (Pl 2, figs 20, 21). Unfortunately these processes neither took up the stain selectively enough nor differed sufficiently in refractive index from the cytoplasm of the neighbouring cells for them to be traced any farther. None of these cells were undergoing mitosis.

These prickle-less, giant basal-layer cells suggest very strongly that the Azan technique provides us with yet another method of demonstrating at least the cell-bodies of non-pigmented, branched cells in white epidermis.

The cells described in this section are almost certainly the 'clear cells' mentioned and figured by Cowdry (1944).

CONCLUSIONS

The facts presented in Part I of this paper concerning the pigmentation of the epidermis support the theory that melanogenesis takes place exclusively within the cytoplasm of dendritic cells which have been shown to be constantly present with approximately the same numerical incidence in all the samples of pigmented epidermis examined. The nucleate nature of these branching elements, questioned by some authorities (e.g. Cowdry, 1938), has been established, and it has been shown that as a result of certain stimuli, e.g. transplantation, they undergo cell division and maintain their type specific cell lineage. In no case was there any doubt as to whether a division stage belonged to a dendritic cell or to an ordinary epidermal cell. These findings conflict with the so called 'transition theory' supported by Bloch (1927, 1929), according to which the dendritic cells are derived from ordinary basal layer cells as a result of functional stimulation and represent an active phase in the pigment-forming function of the latter. The numerical incidence of these cells in conjunction with the numerous processes to which each gives rise provides a system whereby almost every basal layer cell is 'capped' by the button-like end organ of a dendritic cell process. It has been shown that whenever melanin granules are present within the cytoplasm of a basal-layer cell they are invariably located close to the point on the cell wall at which the end-cap of a dendritic cell is applied, although as these cells are traced towards the surface this highly localized distribution is normally lost.

Dendritic cells are the only cells in pigmented epidermis to give a positive Dopa reaction. No instance of an ordinary basal-layer cell giving a positive reaction has been encountered, though the end-caps which make contact with them are often strongly Dopa-positive.

The value of the Dopa reaction in pigment research has frequently been criticized since it was first described by Bloch (1927), on the grounds that a specific 'Dopa oxidase' does not exist and that such cells as leucocytes

which do not play any part in melanogenesis, give a strong positive reaction. The present study is only concerned with the epidermis and within this tissue positive Dopa reactions have only been obtained from dendritic cells.* The end-buttons of these cells gave a particularly strong reaction. Under these circumstances it seems justifiable to regard a positive Dopa reaction as perfectly valid histochemical evidence of the existence of a melanogenic system even though it offers no further information about it. The melanin granules which have been formed within the cytoplasm of the dendritic cells are probably secreted from them by way of the end-buttons and are thus brought immediately into intimate contact with the basal layer cells which may possibly ingest them, a hypothesis supported by the results of tissue culture experiments in which melanin granules have actively been taken up by epithelial cells (Matsumoto, 1918, Smith, 1921, 1925).

The occurrence of extracellular melanin in the intercellular spaces of the epidermis which ultimately is absorbed by, and appears within, the reticulo-endothelial elements of the dermis is explained by the fact that the end-caps of the dendritic cells probably break away from their embraced basal layer cells and disintegrate, thus liberating the contained melanin granules, as the latter travel towards the surface.

The elements so far non-committally designated 'white dendritic cells' have been shown by three different techniques to occur in non-pigmented skin. They so closely resemble the pigmented dendritic cells in all respects, save that of melanogenesis, that they must be regarded as true-breeding variants of the same race of cells. Among all the true-breeding races of cells known to histology it is difficult to imagine two which are obviously so closely related and yet so visibly distinct as are those of the pigmented and non-pigmented dendritic cells.

The dendritic cell must be regarded as a cellular element constantly present within the epidermis, which is therefore a compound tissue composed of cells of at least two distinct races.

Their origin is no longer in doubt. From embryological studies it has been established that in the case of amphibia (Du Shane, 1935, Twitty, 1936) and birds (Dorris, 1939, Eastlick, 1939, Hamilton, 1941) the pigment cells are derived from the neural crest region. More recently and, for the present discussion, decisively, Rawles (1940, 1947) has established that in the mouse too the melanophores (as she calls them) are derived from the same region. Thus the theory that the origin of the dendritic cells is related to that of nervous elements, supported by the work of Masson (1926), has been confirmed.

* In the *white* epidermis of the black and white spotted guinea pig a positive Dopa reaction is never obtained even after traumatic or actinic stimulation (Lewin & Peek, 1941, Ginsburg, 1944). In the case of the so called albino guinea pig, however, the white epidermis of the extremities such as the ears and the soles of the feet may be blackened by such a mild form of stimulation as that provided by cold weather, its dendritic cells will then give a positive Dopa reaction (Ginsburg, 1944), and they may be seen in ordinary split skin preparations. Ordinary white human epidermis would appear to resemble that of the albino guinea pig with respect to its potentiality for melanogenesis and consequent positive Dopa reaction of its dendritic cells.

Sewall Wright (1942) reviewing the work which has been done on the origin and mechanism of distribution of vertebrate pigment cells, suggests that the spotted pattern in guinea-pigs is in part a pattern of arrested migration. This theory was apparently based on the assumption that only the pigmented type of dendritic cell existed. Now that dendritic cells have been shown to be integral components of the guinea-pig's epidermis, irrespective of whether it is pigmented or not, one is entitled to ask whether the future colour pattern of the developing guinea-pig is not determined in the epidermis before the dendritic cells have begun their migration from their presumptive to their definitive positions, since it would appear that they are not melanogenic during the initial stages of the journey (Rawles, 1947).

Apart from the melanogenic role played by the one variant of the dendritic cell race, no evidence can be offered at present concerning the function performed by these cells taken as a whole. The claim of Pautrier, Lévy & Diss (1928), based on a study of the pigmented dendritic cells, that these form a connecting link in a nutritive syncytial system operating between the dermis and the epidermis is certainly very attractive. Most authorities, however, have failed to observe a single downwardly directed dendritic process actually passing into the dermis and no indication of one has been found in the present study. Certainly the dendritic cell system is ideally suited, anatomically, to mediate the spread of a virus type of infection through the epidermis once an infection has started.

Preparations of both types of dendritic cell were demonstrated to Dr W Holmes and to Dr O L Thomas and each, independently, drew the author's attention to their amazing similarity to certain of the glial elements of the central nervous system. This resemblance, which has also been observed by Becker (1927), is particularly striking if we compare white dendritic cells with protoplasmic astrocytes. The latter can only be demonstrated by means of special techniques, most of which involve metallic impregnation, and some of their branches terminate in the form of specific end-organs known as perivascular feet or podies (Penfield, 1932) which are at least superficially similar to the end-cups of dendritic cells. A nutritive function has been ascribed to certain of these glial elements by some histologists (see Ingleby, 1925). Certainly it would appear that both these types of cell are closely related by virtue of their common embryological derivation from the neural crest region.

The relationship which exists between the 'cell of Langerhans' and the dendritic cells described in this paper can only be determined by comparing the white dendritic cells revealed by the gold chloride technique with the 'dark staining bodies' described by Langerhans in his original paper (1868). The account presented in this paper is in almost complete agreement with that of Langerhans with respect to the numerical incidence, size and shape of body and in number of dendritic processes to which the latter gives rise. Moreover, Langerhans clearly describes and figures the terminal buttons. He does not, however, seem to have observed any relationship between the latter

and epidermal cells of the basal layer His cells are described as occurring at a relatively more superficial level in the epidermis than do the dendritic cells The existence of a centrally directed process passing to the dermis, which he described upon slender evidence (on his own admission) has not been confirmed in the present investigation and, finally, even in cases where a rich pigmentation of the epidermis occurred he did not find pigment granules within his cells If due allowance is made for the fact that Langerhans worked with sections while the present investigation has largely been based upon the study of sheets of epithelium then it appears that the main points of difference have arisen through differences of technique and not because the elements investigated were intrinsically different The findings of this investigation support the view that the 'cell of Langerhans' is the same as the (white) dendritic cell

Weddell (1942) in a study of cutaneous nerve regeneration drew attention to the fact that the so-called Langerhans cells are abundant in the skin of the albino rabbit's ear and are revealed by methylene blue staining when the fine regenerating nerve terminals approach them He suggests that these are probably nothing more than modified Schwann cells The results obtained in the present study confirm the presence of elements similar to those described and illustrated by Weddell in the albino rabbit's ear (here described as white dendritic cells), but no indication has been obtained that they lie in series with, or are related to, the Schwann cell elements In conclusion, if the identity of the 'cell of Langerhans' with the white dendritic cells is rejected and the nervous nature of the former is asserted then we are in fact asked to believe that within the epidermis two entirely (functionally) different categories of branching cells exist side by side with a remarkable similarity in form, incidence, location, etc

SUMMARY

1 A reinvestigation of the anatomical basis of pigmentation of mammalian skin has been undertaken based mainly on the study of the pigmented epidermis of the black and white spotted guinea-pig

2 It has been demonstrated that although pigment granules are found in most 'ordinary' epidermal cells of pigmented skin they are not of endogenous origin, but are derived from branched cellular elements which have been called pigmented dendritic cells

3 These branching cells are located at the level of the basal-layer cells of the epidermis From them branches are given off which travel along the inter-cellular spaces between the ordinary epidermal cells, dichotomizing frequently, and ultimately terminate in the form of 'caps' or 'end-buttons' closely applied to the boundaries of ordinary epidermal cells

4 Evidence is presented which indicates that the pigment granules are elaborated within these dendritic cells and are passed on to the ordinary epidermal cells across the end-caps This hypothesis accounts for the well-known initial polar-capping distribution of pigment within the epidermal cells

5 Dendritic cells have a cell-lineage of their own and are not derived from ordinary epidermal cells of the basal layer as a functional modification

6 Branched cellular elements which are similar in all respects to pigmented dendritic cells, save that they lack the melanogenic properties characteristic of the latter, have been demonstrated in the non-pigmented epidermis of man, the guinea-pig and the rabbit. These have been called white dendritic cells

7 It is suggested that dendritic cells (both types) almost certainly fulfil some physiological function in the epidermis other than melanogenesis

8 The relationship between the white dendritic cell and the 'cell of Langerhans' is discussed and it is concluded that they are identical. The theory that these cells have connexions with the nerves of the skin is not supported

9 It is concluded that the mammalian epidermis is a compound tissue composed of at least two distinct cellular elements—the dendritic cells and the ordinary epidermal cells

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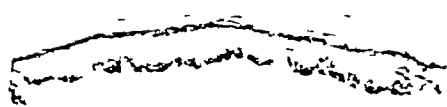
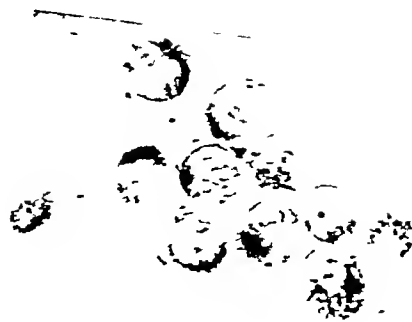
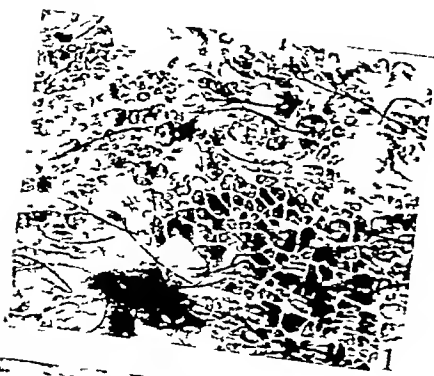
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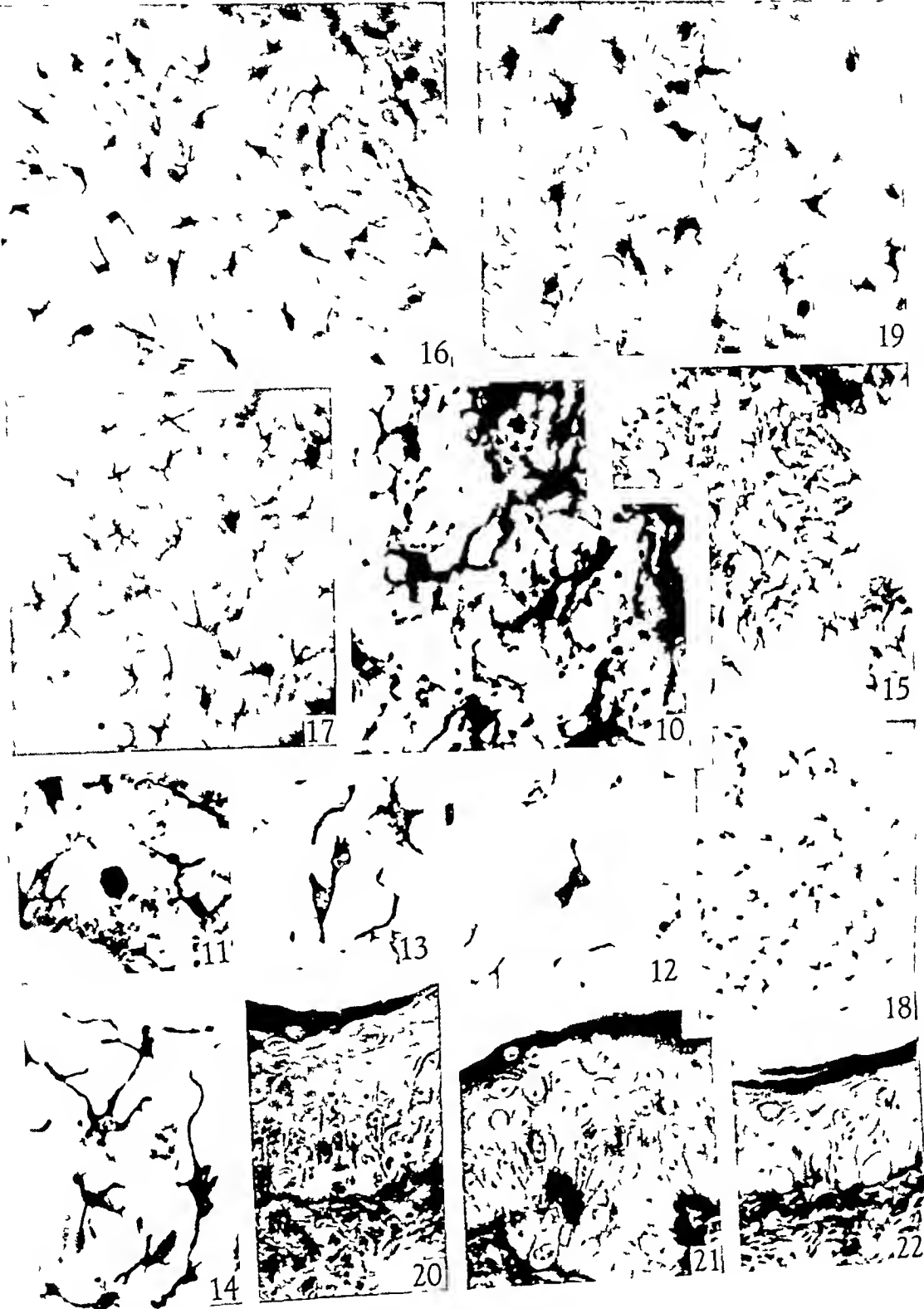
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EXPLANATION OF PLATES

PLATE I

- Fig 1 Whole mount preparation of pure pigmented epidermis from guinea pig's ear, unstained and viewed from the underside to show the characteristic ridging pattern. The 'valleys' are normally occupied by the so called 'dermal papillae' ($\times 20$)
- Figs 2-4 Vertical sections of split black ear skin. The pure epidermis has been separated from the dermis by tryptic digestion and the sections prepared from it lightly stained with Ehrlich's haematoxylin and eosin. Dendritic cells are more prominent than in sections of full thickness skin (Fig 2, $\times 75$, Figs 3 and 4, $\times 335$)





- Fig 5 Isolated ordinary basal layer epidermal cells from pigmented ear skin about 1 hr after teasing out in Ringer's solution. Melanin granules are present *within* the cells as characteristic 'polar caps' located between the nuclear and cellular boundaries. It is evident that cytolysis has commenced ($\times 735$)
- Figs 6-9 Pigmented dendritic cells from surviving preparations of split black ear skin the under sides of which have been lightly scraped and the resultant clumps of cellular material lightly squashed in Ringer's solution. The terminal buttons or end caps of dendritic cell processes can clearly be seen applied to the boundaries of ordinary basal layer cells in Fig 8. Note that melanin granules are present in nearly all ordinary epidermal cells. Fig 9 shows two dendritic cells united by a common process. They may represent the division products of a single dendritic cell which has recently divided, since they possess very few processes (Fig 6, $\times 310$; Fig 7, $\times 220$; Figs 8 and 9, $\times 390$)

PLATE 2

- Fig 10 Pigmented dendritic cells. This is a high power photograph of a preparation exactly similar to that illustrated by Fig 1 after subjection to Dopa treatment for 1 hr. The preparation is unsquashed and the distribution and optical depth of the dendritic cells of ear skin are normal. Ordinary epidermal cells are invisible, but numerous end caps of dendritic cell processes, which are strongly Dopa positive, can be seen ($\times 400$)
- Figs 11-14 Division stages of pigmented dendritic cells. The preparation is unstained and is of pure pigmented epidermis of the ear which had previously been rendered hyperplastic by transplanting it to a recipient area of the animal's chest for 12 days, viewed from the under side. Fig 11 shows a dendritic cell which has retracted its processes and rounded off prior to division. Figs 12 and 13 show later division stages in which daughter nuclei can be distinguished. In Fig 14 two daughter dendritic cells are seen which are still united by a common process although each possesses its own fairly complex system of branches ($\times 270$)
- Figs 15-17 White dendritic cells as revealed by a variant of Garms's gold impregnation technique. Figs 15 and 16 are of the white ear epidermis of the spotted guinea pig, Fig 17 is of the white epidermis of the human forearm. Note that the distribution, number, size and mode of branching is just the same as with pigmented dendritic cells. Fixation with formic acid and lemon juice has, however, caused these cells and their processes to appear more compact and wiry. The nuclei of the ordinary epidermal cells of the basal layer are easily visible. There is absolutely no morphological difference between the white dendritic cells of human epidermis and those of the guinea pig (Fig 15, $\times 100$; Fig 16, $\times 180$; Fig 17, $\times 135$)
- Figs 18 and 19 White dendritic cells of human skin. The preparation is of a Thiersch graft cut from the forearm and stained supravitaly with methylene blue solution in Ringer. The nuclei, cell boundaries and cytoplasmic bridges of the ordinary epidermal cells can be clearly seen in Fig 19. The processes of the dendritic cells have been rendered visible by the presence of deep blue staining granules within their cytoplasm. Compare these white dendritic cells, as revealed by methylene blue staining with those demonstrated by gold impregnation (see Figs 15-17) (Fig 18, $\times 95$; Fig 19, $\times 270$)
- Figs 20-22 White dendritic cells of human skin as revealed by Heidenhain's Azan method. The material was taken from the abdominal region, fixed in formal mercuric chloride and paraffin sections cut at 15μ . These cells are slightly larger than their ordinary basal layer cell neighbours and they have a relatively thicker layer of cytoplasm around their nuclei. 'Prickles' can be seen bridging the spaces between the ordinary epidermal cells but in no case can prickles be seen around the boundaries of the dendritic cells—a diagnostic character of these cells. Processes which arise from the cytoplasm of these white dendritic cells and disappear between the neighbouring cells can be seen in Figs 20 and 21 ($\times 400$)

INTERNODE LENGTH AND FIBRE DIAMETER IN DEVELOPING AND REGENERATING NERVES

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The processes of myelin formation during nerve regeneration have been relatively little studied, it is now known that the normal spectrum of fibre diameter reappears after a nerve has been interrupted by crushing, though not after severance and suture (Gutmann & Sanders, 1943), but there is no information available about the recovery of the internodal distances. In a normal nerve there is a linear relationship between internodal distance and diameter, but it is not known whether this relationship reappears after regeneration.^{*} Investigation of the question should serve to throw light on the unsolved problem of the significance of the nodes of Ranvier. No consistent theory has been put forward to explain the origin and significance of the segmentation of peripheral nerve fibres, though it has frequently been suggested that the nodes play an important part in conduction (see von Muralt, 1946). Thus Hursh (1939) describes an increase of conduction velocity *pari passu* with the length of the limb in cats since internodal length and size of the limb bear a relationship this suggests a possible connexion between internodal length and speed of conduction. Such a connexion has also been suggested by Offner, Weinberg & Young (1940) 'and it is clear that internodal length and width of the Ranvier nodes as well as diameter explicitly determine the functional relationship between velocity and diameter according to the present theory'. However, investigations by Sanders & Whitteridge (1946) of regenerated nerves, in which all the internodal segments were short, showed no relation between internodal length and conduction velocity. The function of the nodes therefore remains quite uncertain their origin is equally obscure. The observations of Speidel (1932) suggest that the spacing of the Schwann cells controls internode lengths, alternatively it has been suggested that the segmentation is determined by surface tension dividing the myelin into long droplets (Young, 1945).

To test these theories we have made estimations of the relationship between internodal distance and diameter in nerves of rabbits under the following conditions (1) in normal adult animals, (2) in adult animals during the stages of nerve regeneration, (3) in young animals at various stages of growth, (4) in young animals during the stages of nerve regeneration.

* After this paper had gone to press the paper by Hiscoc, H B, 1947, The distribution of nodes and measures in normal and regenerated nerve fibres, *Anal Rec* 99, 417 was received. She showed that after 17 weeks of regeneration of the nerves of adult rats the internodes of all fibres are of about the same size, 300 μ , irrespective of calibre. This length was also found on the fibres of young rats.

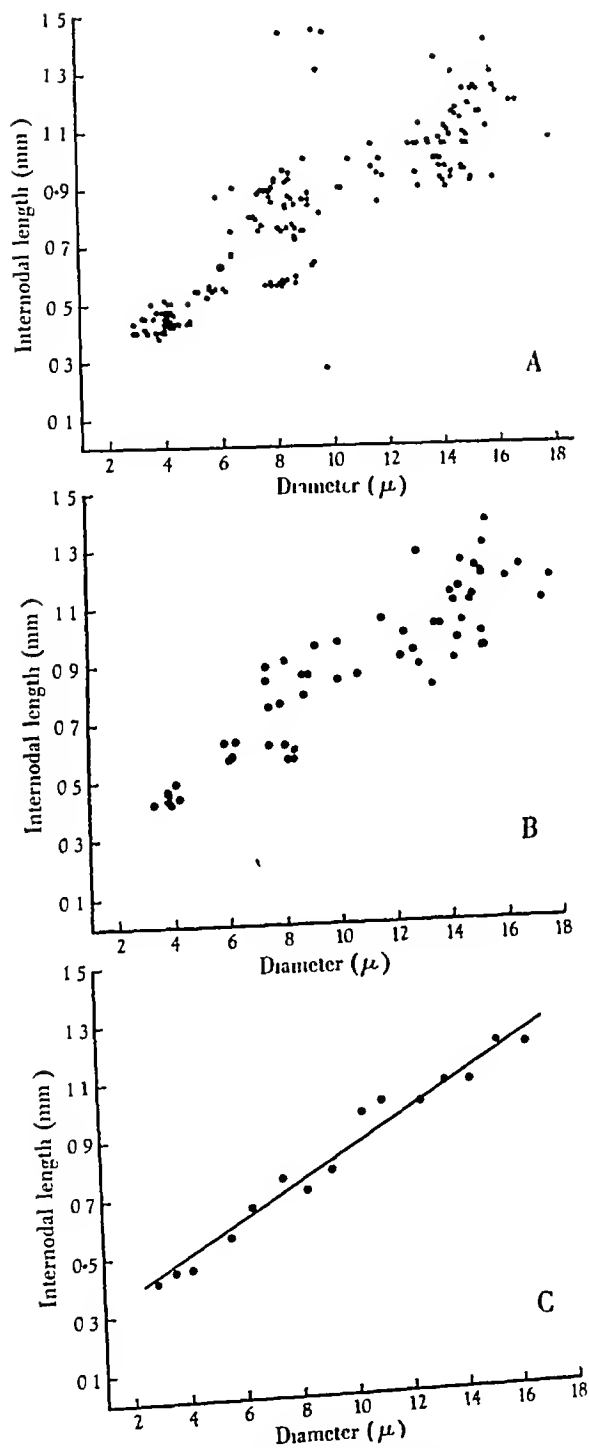
We shall not here discuss the nature of a node of Ranvier but consider that it is definable as a place where the myelin sheath is interrupted, leaving the axon bare. The ends of the myelin on either side of the node may take various forms but, in the adult at least, they are smoothly rounded and covered by an inturning of the neurilemma, making the cementing disc of Cajal. The axon narrows at the node, but we have not been able to recognize the transverse membrane across the axon postulated by von Muralt (1946). Many of the following measurements depend on the recognition of nodes and the above criteria serve to distinguish nodes from accidental post-mortem breaks (not smoothly rounded and without cementing disc) or from Schmidt-Lantermann incisures (oblique and without smoothly rounded ends). Nevertheless, the recognition of nodes is not always easy, especially in young animals and on small fibres. The myelin may taper off gradually instead of making a sudden inward turn and the neurilemma is then difficult to discern. However, in such small fibres the axon surface is usually bare for a considerable stretch (Pl. 1, fig. 2), making it easier to recognize the node.

METHOD

All experiments were made on rabbits. The nerves were exposed and crushed with fine smooth-tipped watchmaker's forceps under nembutal and ether anaesthesia, and the animals allowed to survive for periods up to 486 days. Nerves taken from these animals were first stretched on cards, then placed in 4% formaldehyde in 0.9% NaCl and so stored until required for staining with solutions of 1% osmium tetroxide in 0.9% NaCl. After staining the nerves were stored in glycerine to which one-third of water was added, in this solution the dissociation of the fibres became easier.

For study of internode lengths it is necessary to obtain single fibres dissociated for a distance of many nodes. With the above method of fixation and maceration it proved possible with care to isolate and mount lengths of more than a centimetre, the longest fibre obtained showing 33 nodes. The nerves were dissociated under a dissecting microscope with a pair of very fine needles, until single fibres were obtained. Great care was taken to minimize damage or stretching of the fibres. Small fibres (under 5μ in diameter) were very difficult to separate, and to avoid the danger of injury or stretching it was found convenient to leave them attached to thicker ones. After the nerve fibres had been isolated from the bundles they were removed with the aid of a dissecting needle to a slide on which a large drop of clove oil, or creosote, had been placed. After a number of fibres (10-20) had been so transported and placed in the desired position, the creosote or clove oil was drained off, the remainder blotted with filter paper, and a coverslip placed with Canada balsam.

When a drop of highly viscous balsam is placed on the coverslip over the fibres the flowing of the balsam between the slide and coverslip causes the fibres to move out of position. This was prevented by warming the coverslip and the balsam before covering the fibres. The balsam then flowed easily



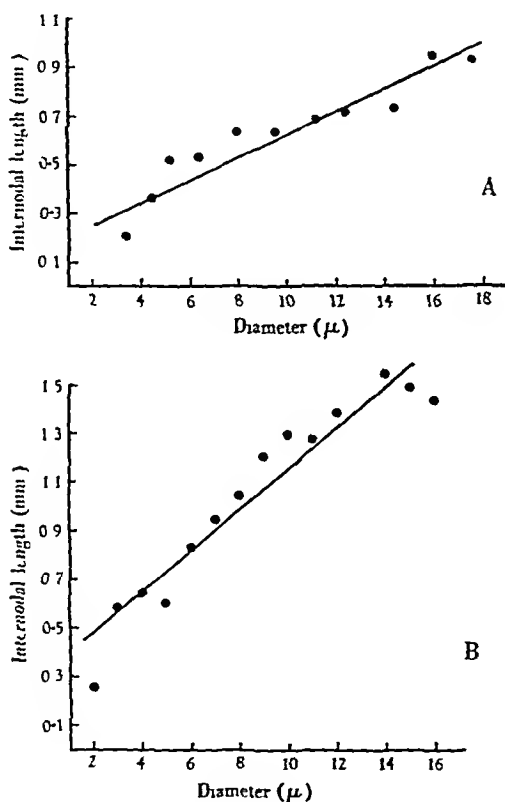
Text fig 1 Fibres from peroneal nerve of four normal adult rabbits A, individual internodes, B, mean internodal length and diameter per fibre, C, grouped by diameter

in Text-fig 1C is that obtained by the use of the usual methods of linear regression (Fisher, 1944) and if d is the average diameter of individual internodes in μ and l their average internodal length in mm,

$$l = bd + a,$$

and b is $+0.059 \pm 0.001$ and a is $+0.24 \pm 0.007$

A linear relationship between internodal length and fibre diameter has been found by earlier workers also. Key & Retzius (1876) gave some measurements made on myelinated fibres in dog, rabbit, finch, pike and man, stating that



Text fig 2 Previous data on internode lengths in nerves of rabbits, plotted as in Text fig 1C
A from Key & Retzius (1876), B from Kubo & Yuge (1938)

these data showed a relationship between fibre diameter and internodal length. Text-fig 2A shows the data which they give for the rabbit, plotted in the same way as the results of the present measurements given in Text-fig 1C. The calculated regression line for their data has a slope of $b = +0.042$ and cuts the l -axis at $+0.21$ mm. The data of Kubo & Yuge (1938) from the rabbit are shown plotted in the same way in Text-fig 2B, the constants being $b = +0.082$ and $a = +0.32$ mm. Therefore all the lines meet the l -axis close together but the

data of Kubo & Yuge show much faster increase of internodal length with diameter than do those of Key & Retzius and the present experiments

Irregularities of node length along the fibres

In any one fibre successive internodes have, as a rule, approximately the same length. Occasionally, however, fibres are found in which one or more of the internodes is very much longer or shorter than the rest of the segments in the fibre. In Text-fig 1A, it will be seen that there are a few points which stand widely apart from those representing the majority of internodes for any given diameter. In one case (Table 1(a)), a fibre approximately 9μ in diameter showed one internode of 1.44 mm and seven between 0.63 and 0.76 mm. Excluding the long internode the mean internodal length of this fibre was 0.71 mm, the long segment is therefore almost exactly twice as long as the average internodal distance. In another case (Table 1(b)), of a fibre of average

Table 1

Mean internodal length of the whole fibre (mm)	Internodal length (mm)	Mean diameter of each internode (μ)	Mean diameter of fibre (μ)
(a) 0.795	0.64	9.4	8.7
	0.72	8.2	
	0.63	9.3	
	1.44	9.0	
	0.72	8.7	
	0.76	8.4	
	0.72	7.6	
	0.73	8.7	
(b) 0.848	0.58	9.8	9.8
	0.69	12.0	
	1.47	8.9	
	0.75	9.0	
	0.75	9.5	
(c) —	1.47	18.0	—
	0.42	11.0	
	0.41	11.0	
	0.70	11.0	
	1.48	18.0	
(d) 1.05	1.00	15.1	13.7
	0.46	13.5	
	0.27	9.8	
	0.43	12.4	
	1.10	14.3	
	1.05	12.9	
	1.03	14.3	
	1.05	13.2	
	1.05	14.9	
	1.05	14.9	

diameter 9.8μ , five successive nodes were measured, here again there is a 'long' internode, the average length (excluding the long node) is 0.69 mm, the long internode has a length of 1.47 mm, almost double that of the others.

There were also cases of short nodes among long ones (Table 1(c) and (d)) and here the abnormal nodes have lengths which are about one-half, and one-quarter that of the normal. The diameter varied irregularly in such fibres.

and in some cases the short internodes were also narrow. The probable explanations of all these irregularities will be discussed on p. 129.

Irregularities of diameter along internodes

The fibres usually swell out close to the nodes. Table 2 shows ten measurements of diameter taken at approximately equal distance along successive internodes.

Table 2

Mean internodal length (mm)	Internodal length (mm)	Diameter (μ)										Mean diameter (μ)	Mean for fibre (μ)
0.944	0.93	16	14	14	14	13	16	13	13	15	16	14.4	12.68
	0.85	16	13	9	12	11	11	11	11	11	12	11.7	
	0.87	13	12	13	11	11	11	10	12	12	14	11.9	
	1.00	14	12	10	13	12	10	10	11	11	14	11.8	
	0.97	14	10	11	11	12	12	10	10	10	15	11.5	
	0.97	14	13	14	14	15	14	12	13	14	17	14.0	
	1.02	15	11	10	13	14	14	15	14	13	16	13.5	

(2) *Internode lengths in adult regenerated nerves*

The new fibres which invade the peripheral stump during nerve regeneration are at first thin and non-myelinated, and only later thicken and acquire myelin sheaths (Hentow, 1933, Young, 1942, Gutmann & Sanders, 1943, Rexed & Swensson, 1941). We have been able to confirm the observation of Sherrington (1895), Rexed & Swensson (1941) and others that the earliest myelin forms a continuous coat over the surface of the fibre. Nodes appear as the fibres become larger but there is little information about the time of their appearance, or whether they are formed at distances corresponding to those found on the same fibres before the lesion. The experiments described in this section were undertaken in order to investigate this question.

The peroneal nerve of rabbits was crushed with fine watchmaker's forceps at a point about 80 mm above its entry into the peroneal muscles. Following the operation the animals were allowed to survive for various periods of time and the nerves were then excised, fixed, stained, teased, and their constituent fibres measured as previously described. The work of Gutmann & Young (1944) has shown that after crushing the peroneal nerve of the rabbit at this level fibres return to the muscle after 31 days, and recovery of the spreading of the toes occurs at about the 53rd day. In addition, Gutmann & Sanders (1943) have shown that when a nerve is crushed at 80 mm from the muscle myelinated fibres are found throughout the peripheral stump after 60 days of regeneration, although they are all small. It was therefore thought unprofitable to study the relationship between the internodal length and fibre diameter before a lapse of 60 days, and nerves were accordingly removed after 60, 90, 160, 190, 456 and 486 days of regeneration.

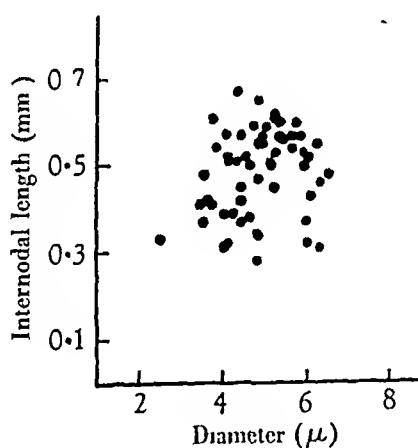
60 days of regeneration after crushing

Fibres of a nerve 60 days after crushing present a very irregular appearance, but some undoubted nodes are seen. Regions without apparent nodes may follow parts of the same fibre in which the nodes are clearly marked (Table 3)

Table 3

Table 3													
Mean internodal length (mm)	Internodal length (mm)	Diameter (μ)										Mean diameter (μ)	Total mean diameter (μ)
0.558	0.12	5	5	5	5	5	5	5	5	5	5	5.0	5.3
	0.70	6	6	5	6	7	7	6	6	6	6	6.1	
	0.54	5	4	5	5	6	7	6	6	6	6	5.6	
	stretch of 2.8 mm without nodes										5.4		
	0.56	6	6	5	6	6	6	5	4	5			

Moreover, some fibres were found in which, over the length studied, no nodes were seen at all. Sherrington (1895) described similar irregularities of fibres and the absence of distinct nodes in the femoral (anterior crural) nerve of a cat severed 74 days previously. Text-fig. 3 shows the length of individual internodes



Text fig. 3 Internodes from peripheral stump of peroneal nerve crushed with smooth forceps 60 days previously

assessed in this way, plotted against their diameter. The fibres measured have a diameter ranging from 2 to 7 μ and internodal lengths varying from 0.3 to 0.7 mm. As the range of fibre diameter is so small it is not profitable to fit a regression line to these data. The internodal length of the larger fibres is shorter in the regenerating nerve than in normal adult fibres of corresponding diameter.

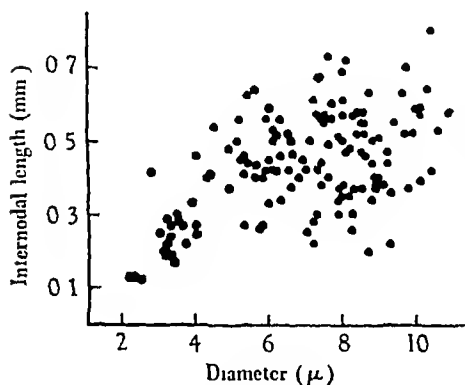
90 days of regeneration after crushing

Many of the fibres in a nerve 90 days after crushing still have an irregular appearance and sometimes there are long stretches of fibre in which there is

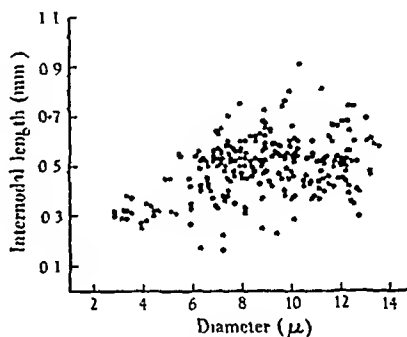
a myelin sheath but no nodes Text-fig 4 shows measurements of individual internode lengths plotted against their corresponding diameters. Fibres ranging in diameter from 2 to 11μ are present, as described by Gutmann & Sanders (1943), and the internodal length shows a wide scatter, the internodes are often very short even on the larger fibres. As at 60 days, there may perhaps be a slight tendency for increase of internode length with diameter, but nothing like the normal relationship has reappeared.

160 days of regeneration after crushing

In this nerve all the fibres examined showed nodes along their whole length, no stretches of unsegmented myelin such as appeared after 60 and 90 days were seen. Text-fig 5 shows internodal length plotted against diameter in the



Text fig 4 Internodes from peripheral stump of nerve crushed 90 days previously



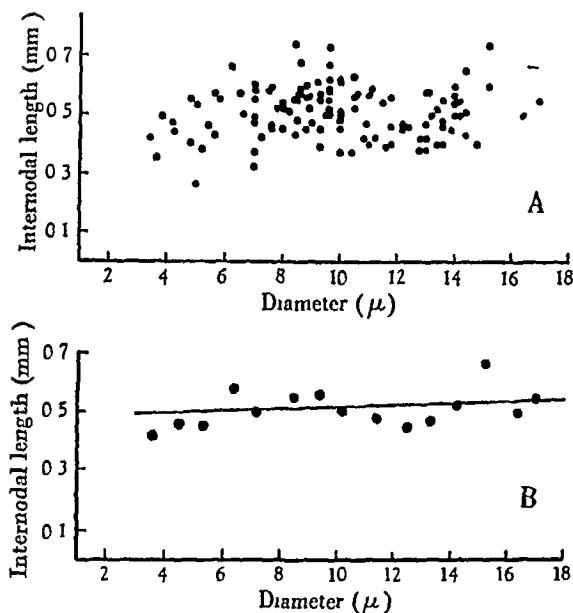
Text-fig 5 Internodes from peripheral stump of nerve crushed 160 days previously

case of 357 internodes from 35 fibres taken from this nerve. The range of diameters represented is less than that found in the normal nerve. Internodes vary in length from 0.10 to 1.05 mm, but the great majority of the measurements lie between 0.25 and 0.75 mm. There are altogether 19 internodes between 0.1 and 0.3 mm in length, several of them little above the lower figure, and we must therefore conclude that these very short periods are not uncommon, on the other hand there are only two internodes longer than 0.8 mm. Over the range 0.1–0.8 mm the internode lengths are scattered in approximately a normal distribution, with mode at 0.5 mm. As at earlier times, there is still a wide range of internode length for each fibre diameter. Comparing the figure with the corresponding one for the normal nerve the following differences are seen: (1) The normal nerve contains a substantial number of internodes of considerably greater length than those seen in the 160 days peripheral stump. The normal fibres have internodes measuring up to 1.30 mm, while in the 160 days stump there are very few over 0.70 mm. (2) Fibres of similar diameter have shorter internodes in the 160 days stump. (3) The correlation

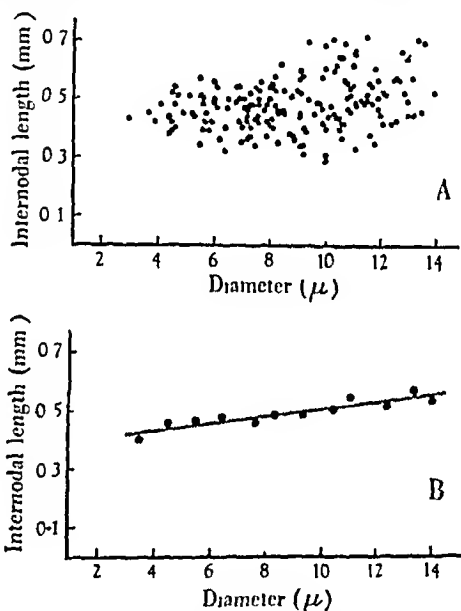
between internodal length and diameter is much less close than in the normal nerve

456 days of regeneration after crushing

Fibres from the peripheral stump of a nerve taken 15 months after interruption by crushing showed no greater internodal length than in earlier stages. Text-fig 6A shows the individual internodes plotted against their diameters. The largest fibres recorded are $16\text{--}17\mu$ in diameter and internodes vary in length within a narrower range than before, from 0.26 to 0.74 mm, the majority measuring from 0.35 to 0.65 mm, the mode being at 0.5 mm. As after the shorter time of regeneration, there is a great variation in the



Text-fig 6 Peripheral stump of nerve crushed 456 days previously A, individual internodes, B, grouped by diameter



Text-fig 7 Peripheral stump of nerve crushed 486 days previously A, individual internodes, B, grouped by diameter

internodal lengths and these are distributed regularly about the mode. At any given diameter nodes of a variety of lengths are to be found. Text-fig 6B shows the internodes grouped according to diameter classes, and the regression line calculated for the points in this graph is nearly parallel to the base line, b being only $+0.002$ and the line cutting the l -axis at $+0.49$ mm.

Even after this long period of regeneration, therefore, the internodal length remains short, in fact it is comparable over the whole range of diameters with the internodal length of normal fibres of $3\text{--}5\mu$ in diameter. Moreover, a considerable variability of internode length is still found.

486 days of regeneration after crushing

Another nerve, examined 486 days after interruption, fully confirms the results found at 456 days. Text-fig 7A shows the internodal length plotted

against diameter of individual segments and in Text-fig 7B the measurements are grouped into diameter classes and their mean internodal length plotted. The regression line shows a very slight increase in the internodal length with diameter, having a slope of $b = +0.012$, and cutting the l -axis at $+0.38$ mm. In this animal, as in the previous one, the nerve contains only occasional macrophages and the fibres have an appearance suggesting a complete regeneration in everything except internodal length. At this time the fibre diameters have increased in such a way as to reconstitute the normal fibre spectrum almost exactly (Gutmann & Sanders, 1943), yet the internode lengths remain abnormally short. Pl 1, fig 1 shows two normal nerve fibres (A and B) for comparison with the regenerated fibres (C and D) which have short internodes.

(3) Relation between internodal length and fibre diameter in growing rabbits

In an attempt to throw further light on the relationship between internodal length and diameter an investigation has been made of the conditions during development. Three animals from a single litter were used and the length of the femur was measured each week, animals being killed at 2, 4 and 12 weeks. It was found that the femur length of the rabbits at 2 weeks was approximately one quarter, at 4 weeks one-half and at 12 weeks three-quarters of that in the mother of the litter.

In the youngest of these animals some of the fibres presented a complete myelin sheath and where this was present it was always divided into segments by nodes. However, the shape of the end of the myelin differs from that of an adult segment, and usually a rather long stretch is left naked at the nodes (Pl 1, fig 2). It is not always easy to recognize the nodes on these smallest fibres and we cannot exclude the possibility that in the very first stage of its production the myelin is not divided by nodes. Kappers, Huber & Crosby (1936) report that 'the myelin first appears during development as a thin continuous layer with no nodes of Ranvier'. Others have described nodes from the beginning and we are not able at present to say whether Kappers's observation is true of some stage earlier than we have observed. Even non-myelinated fibres have a layer of orientated lipid material (Schmitt & Bear, 1939) and this is presumably not segmented, but the nodes appear at least soon after an osmium-staining layer can be seen.

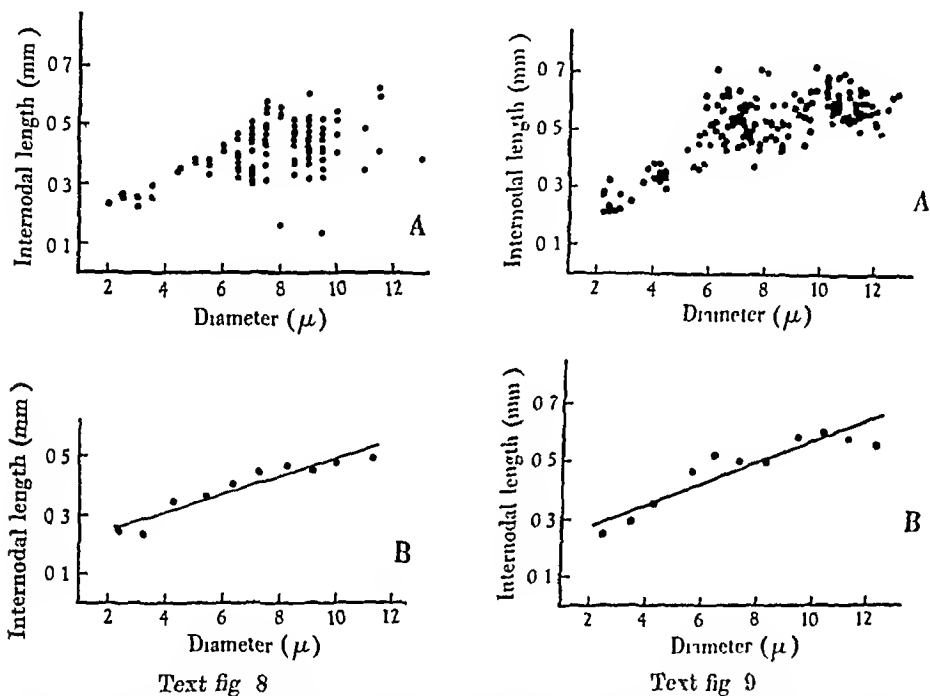
Some fibres showed an undulating outline of the myelin, and in others the myelin was broken into droplets of different sizes (Pl 1, fig 2), sometimes with a 'neck' of myelin between two beads. Previous authors, Vignal (1883), Westphal (1894), Bardeen (1903), have described similar appearances in the myelin at different stages of growth. They interpreted the early appearance of droplets along the fibres as showing growth into larger droplets, which finally join each other and grow towards the nodes.

These curious appearances were observed on many fibres from several young animals. Since the nerves had been prepared in the same way as the normal ones, previously described, it can only be concluded that these are stages in

the formation of the myelin (see p 126) The nerves taken from animals older than 4 weeks showed these phenomena very rarely It is obviously impossible to measure the internode length in a fibre divided in this way and our measurements have therefore been restricted to cases where segments resembling normal internodes were found

2-week old rabbit

In Text-fig 8A the lengths of individual segments of the peroneal nerve of a 2-week old rabbit are shown plotted against their corresponding diameters. The lengths range from 0.1 to 0.6 mm, but the two lowest points are so isolated as to suggest error and remaining points lie between 0.25 and 0.60 mm. The



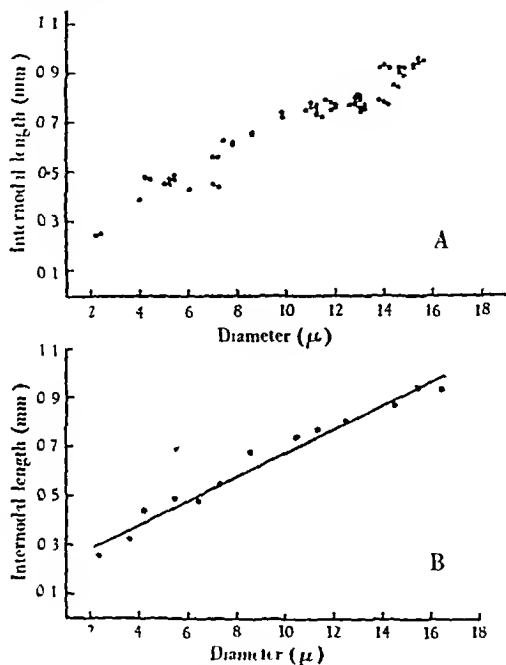
Text figs 8 and 9. Fibres from peroneal nerves of litter mate rabbits, 2 and 4 weeks old respectively. A, individual internodes, B, grouped by diameter.

internodes were also classified into diameter groups, as was done with the data of the adult animals (Text-fig 8B). From these data a correlation line has been calculated, it cuts the l -axis at 0.22 mm and the value of $b = 0.030$. As in the case of the regenerating nerves an outstanding characteristic is the variability of the internode lengths at a given fibre diameter and this makes the significance of any trends doubtful. However, Text-fig 8B suggests a distinct tendency for internodal length to increase with diameter, but at a much slower rate than in the adult. Fibres of a given diameter therefore have shorter internodes at this stage than later.

4-week old rabbit

In Text-fig 9A internodal lengths are shown plotted against diameters in the case of a 4-week old rabbit. The points are much less scattered than at

2 weeks and in particular the larger fibres do not show occasional short nodes. The line suggested by the points shows a more definite upward trend than in the previous animal. The maximum diameter is little larger, but the nerve contains more of the larger fibres, moreover the internodes are longer for any given diameter, reaching as much as 0.72 mm. Text-fig 9B shows the regression line for the data grouped by diameters. This line has a slope of $b = +0.036$ and cuts the l -axis at $+0.25$ mm.



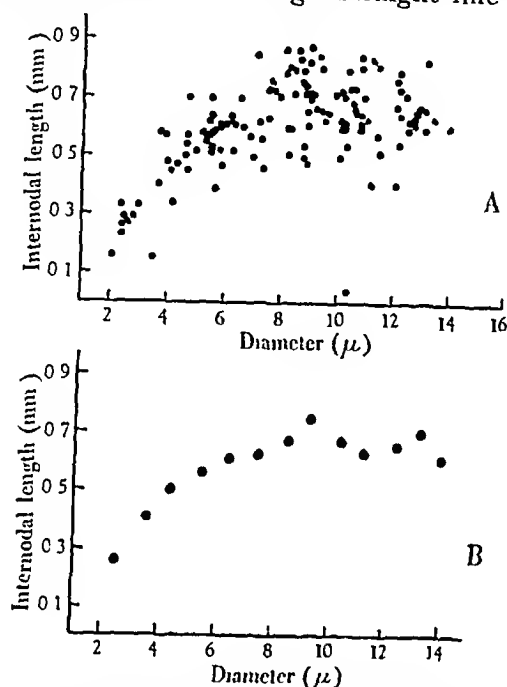
Text-fig 10 Fibres from peroneal nerves of rabbit, 12 weeks old
A, individual internodes, B, grouped by diameter

12-week old rabbit

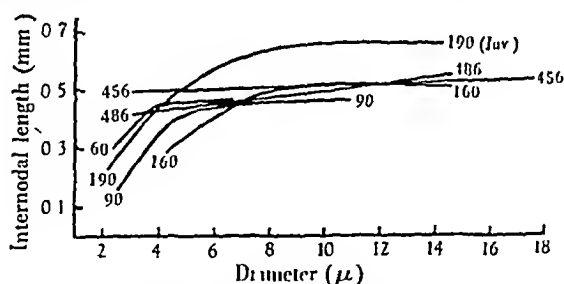
In Text-fig 10A the diameter and internodal length of a number of internodes from a 12-week old rabbit are shown plotted against one another. For each diameter there is an appropriate internodal length and in fact the closeness of correlation between diameter and internodal length in this animal approaches that seen in the adult nerve. However, although the diameters range up to 17μ the longest internode is only 1.07 mm. Text-fig 10B shows the results after grouping by diameter, the regression line of internodal length on fibre diameter has a slope of $b = +0.048$ and cuts the l -axis at $+0.25$ mm. It is therefore steeper than in the two previous animals, but less steep than in a normal adult animal. Text-fig 10B may also be compared with the regression line calculated for the data of Key & Retzius for an 'adult' rabbit (Text-fig 2A) which cuts the l axis at a similar point and has a similar slope.

(4) *Regeneration after crushing the nerve of a young animal*

Text-fig 11A shows the internodal length of individual segments plotted against their diameter in the case of a nerve crushed when the animal was 11 days old and then allowed to regenerate for 190 days. Fibres varying in diameter from 2 to 14μ were present, as in the adult nerve 160 days after crushing, and the internodal length varied from 0.14 to 0.90 mm. It is obvious that it would be impossible to fit a single straight line to this collection of



Text fig 11 Fibres of peripheral stump of peroneal nerve of rabbit crushed when the animal was 11 days old and then allowed to regenerate for 190 days. A and B as before



Text fig 12 Lines fitted to data grouped by diameter for fibres of peripheral stump at various times after crushing. All animals adult except that marked 'Juv', curved lines fitted by eye where necessary

points, and this is even more clearly brought out by the plots of mean internodal distance against mean diameter per diameter group (Text-fig 11B). Internodal length showed an increase with diameter over the range 2–8 μ , but not above this.

In this young animal the larger fibres had considerably greater internodal length than fibres of comparable diameter in the adult nerves regenerated for a similar time. In Text-fig 12 the curves which can be approximately fitted to the

graphs of mean internodal length against mean diameter per diameter group in the case of all the regenerating nerves investigated are shown plotted together. The striking fact about these data is the close correspondence both in slope and position between the regions of the graph related to the larger fibres for all the adult animals, whether the nerves were taken after 60, 90, 160, 456, or 486 days of regeneration. In the case of the animal in which the nerve was crushed when it was young, however, the internodal length evidently became considerably greater, the line becoming horizontal at $l=0.70$ mm instead of $l=0.45$ mm.

DISCUSSION

(1) *The early stages of myelin formation*

Although much remains uncertain it is possible from these data to give a provisional general theory about the origin and distribution of the nodes of Ranvier. During development it seems that the myelin is segmented from its first appearance, it is possible that at a very early stage it forms a continuous cylinder (see Westphal, 1894, and p. 121), but by the time the sheath is readily visible it is already divided by nodes. Since the Schwann cells are spaced out along the fibres before myelination it might be that they are the determining factor in this initial segmentation. Speidel (1932) shows how during the outgrowth of regenerating nerve fibres short internodal segments are formed around each Schwann cell nucleus, and later become elongated. Such short segments may be almost spherical at first, but in normal development they are probably more elongated at their first formation and in view of all the evidence that the myelin tends to break into segments under surface tension (Young, 1944, 1945) it is tempting to look to that force for an explanation of the initial subdivision. The myelin is certainly able to behave as a fluid and indeed is added to the sheath in the form of round drops. Presumably, therefore, during development there is a continual readjustment of this liquid coat covering the axon surface and it is possible to imagine conditions in which such a viscous liquid, enclosed between an inner and outer cylinder, would break into stable segments about 0.25–0.30 mm long. It is very remarkable that this value reappears repeatedly as the internode length of the smallest developing regenerating or adult fibres, shorter internodes have been reported only in a few cases from the early stages of regeneration when it is difficult to be sure of the status of the divisions seen in the fibres (p. 119).

The whole internode once formed was compared long ago by Ranvier (1878) with a liquid drop, enclosed within the Schwann cell as is the fat droplet within a fat cell. It has been suggested (Young, 1944) that the shape of the ends of the nodes would be explained if the myelin wets the surface of the axon but not that of the tube in which it runs. Early in development the axon is left bare for long stretches at the nodes presumably because the myelin tends to remain restricted by surface tension around the Schwann cell nucleus where it has originated. This tendency is opposed by the growth pressure of the axon which, if the neurilemma is sufficiently resistant to stretch, will press out the myelin

towards the nodes. Early in development the neurilemma is very thin and therefore stretches readily and the nodes are at first long. As the tube wall becomes more rigid the myelin is pressed against it and eventually fills up the whole space, producing the typical adult form of the node. At some stage the material of the neurilemma, turning in over the myelin at the node, becomes thickened to make the 'cementing disc' which adds to the stability of the node and perhaps retains the myelin in place.

This theory may prove far too simple to explain all the facts, but it seems to fit those at present available. Further information may show that the myelin is not all produced near the Schwann cell nucleus and we certainly know too little to say how its lamellae are laid down. Round drops are often seen in various parts of developing fibres, especially near the nucleus and at the ends of the internodes.

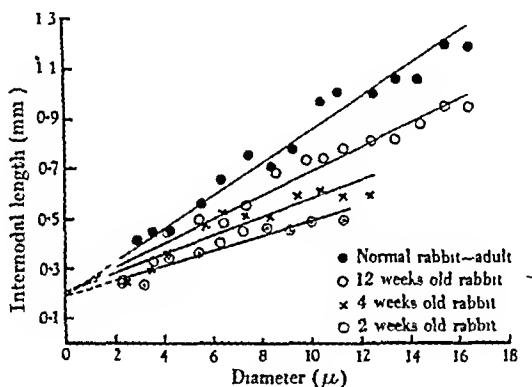
The fact that the myelin is observed to subdivide into shorter segments (ovoid droplets) during the period of 2-4 weeks after birth (p. 121) is presumably a further manifestation of its inability to cover the increasing surface. Later, when more myelin is added, these droplets are squeezed together and rejoin, so that from 4 weeks onwards a regular segmentation is observed along most of the fibres.

(2) *The linear relationship between internodal length and fibre diameter depends upon stretching of the nerve after myelination*

If the above analysis of the beginnings of myelination is correct the linear relationship in the adult will follow if the segments are later stretched by growth in length of the limb. Little is known about the way in which growth in length is achieved in peripheral nerves, but Weiss (1941) has suggested that once nerves have made connexion with the muscles or other organs they are later increased in length by a 'towing' action produced by the growth movements of the neighbouring parts. This would introduce another factor into the conditions controlling the stability of the myelin and might stretch out the already formed elongated myelin drops. Otuka (1940) found in the splanchnic nerve of the cat that those fibres which will become largest are the first to myelinate. It is probable that a similar process occurs in the somatic nerves of all mammals, those fibres which will become larger (i.e. somatic motor and annulo-spiral proprioceptor fibres) becoming myelinated at an earlier age than the eventually smaller sensory and motor fibres. It follows that adult large fibres have been fully myelinated during a greater period of growth in length of the limb than adult small ones.

If, when myelin is first laid down, the nodes are formed with the same spacing on all fibres, then the later increase in length of the nerve will ensure that large fibres come to have longer internodes than small fibres. The linear relationship between internodal length and fibre diameter found in adult animals would then be a result of the growth of the limb in length and need not necessarily have any particular significance for conduction.

Evidence that initial spacing of the nodes is the same on all fibres can be seen from Text-fig 13, where the regression lines of internodal length on diameter found in animals of ages 2, 4 and 12 weeks and the adult are shown plotted on the same graph. These lines show a very close approach to a meeting point at fibre diameter about 1μ and internode length 0.25 mm. The regression line calculated from the data of Key & Retzius (Text-fig 2A) meets the same point and so approximately does that for Kubo & Yuge (Text-fig 2B). The critical diameter above which all fibres are myelinated (see Duncan, 1934) is 1μ and this is therefore presumably the diameter at which a myelin sheath first appears on a fibre during development. The above data indicate that the spacing of the nodes is the same on all fibres when they have a diameter of 1μ whatever diameter they may ultimately attain, this uniform initial length itself strongly suggests that there is a physical factor determining the segmentation.



Text fig 13 Internodal lengths and diameters for rabbits of various ages, grouped by diameter

Text-fig 13 also presents further confirmation of the hypothesis that the linear relationship seen in the adult animal results from growth in length of the nerve after myelination. On this figure the four regression lines make approximately equal angles with one another, each successive line representing the same increment in slope as the one before it. The young animals for which the lines were plotted had femora which were approximately $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$ times as long as the adult femur. The steepness of the regression lines in these animals increases in a similar way, as would be expected on our hypothesis.

Some interesting consequences follow from a wider application of this hypothesis. Thus, if we were to compare the length of internodes on large fibres of similar diameter in nerves taken from two parts of the body in which the relative growth rate following birth is markedly different (for example the facial nerve and the sciatic), we ought to find that the nerve in the more rapidly growing region has longer internodes than the nerve from the more

slowly growing region Preliminary measurements show the expected relations in nerves of Man and of fishes

(3) *The number of internodal segments in a fibre is constant after myelination is complete*

There is evidence that, as our hypothesis requires, during the growth of the animal the number of internodes upon any one fibre remains constant Boycott (1904) and Takahashi (1908), who measured the length of internodes in fibres of all diameters in frogs of different sizes, found that the graph of 'average internodal length' for fibres taken from the sciatic nerve, plotted against body length, was practically identical with the graph of sciatic nerve length plotted against body length By contrast the graph of the number of internodes (sciatic length/average internodal length) against body length was a line parallel to the latter axis

A similar condition obtains in the rabbit The internodal distance of fibres of small diameter of a normal adult animal or of young animals is 0.25 mm, whereas the largest adult fibres show an internodal distance of 1.30 mm, approximately six times as great Using femur length as an index of the growth of the limb we also find from a few measurements that the limb increases in length by a factor of nearly six times during the period of growth from birth to the adult phase (femur length at birth, 20 mm, in adult, 120 mm) Assuming that the nodes are initially spaced 0.25 mm apart in an animal of a femur length of 20 mm, in a piece of nerve equal in length to the femur there will be approximately 100 internodes, and this figure remains constant throughout life

(4) *The linear relationship between internodal distances and fibre diameter*

We are now in a position to discuss the discrepancies between the data on internodal length presented by previous authors The regression line of internodal distance on fibre diameter plotted from Kubo & Yuge's data (1938) has a much steeper slope than that obtained either from our present data or that calculated from the fragmentary data given by Key & Retzius (see p. 115) A possible explanation of this discrepancy may be found in the observations of Boycott (1904), Takahashi (1908) and Hatai (1910) that internodal length increases towards the periphery in frogs, presumably because the growth rate there is high It may be that fibres of a given diameter taken from the periphery of the limb would show more widely spaced nodes than pieces of the same nerve taken more proximally, and a regression line plotted from data obtained from fibres taken at a variety of levels might be steeper in slope than one derived from measurements of internodes taken from a single level only Kubo & Yuge (1938) found internodes in mammals up to 1.96 mm long on fibres 16 μ in diameter, much longer than we have found in the rabbit Similarly, in the toad, they found internodes up to 3.7 mm in length, distances far greater than those found by Boycott (1904) at the proximal ends of frog nerves Unfortunately,

Kubo & Yuge do not state from what level on the nerve their specimens were taken, or whether from a number of levels

By contrast a regression line calculated from the data of Key & Retzius is less steep than that given by our own data from adult animals, though their line corresponds closely to that given by internodes taken from an immature animal 12 weeks old. Key & Retzius (1876) do not mention the age of the animal from which their fibres were obtained: it is clear that it cannot have been very young since fibres up to 17μ in diameter were found.

From the present data and those of the above-mentioned authors it can be seen that there is a linear relationship between internodal length and fibre diameter for all vertebrates for which measurements have been recorded. The slope of the regression line expressing the relationship may vary even for the same nerve and species as a result of such factors as size, age, and level from which the specimens of nerve are taken.

(5) *Irregularities in the size of the internodes are sometimes found in normal nerve fibres*

As previously mentioned, abnormally long or short internodes are sometimes found in normal nerve fibres. In some cases one individual segment measured twice the length of any other segment in the same fibre (see Table 1). It is probable that such segments are formed by the disappearance of a node, producing a single segment twice as long, with approximately the same diameter as the rest of the fibre. Speidel (1932) described a case of end-to-end anastomosis of internodal segments. This seems to correspond to that of our two first examples (Table 1(a), (b)), where the long segments have approximately twice the length of the average measurements of the remaining internodes. Such changes would agree with the hypothesis put forward above, about the development of the myelin. It was suggested that towards the end of the development of a fibre the myelin becomes pressed out away from the Schwann cell body, tending to fill up the space at the nodes. It seems likely that in some cases this would lead to disappearance of the node altogether.

In the remaining examples of irregularities we find the opposite phenomena. On a fibre with long internodes there appear one or several smaller internodes. The diameter may be either the same as, or smaller than, that of the remainder of the fibre. The explanation of this condition may be found in the 'spontaneous' degeneration of myelinated pathways described by Speidel. For instance, in one of the fibres mentioned on p. 116, an internode seemed to have degenerated and been replaced by three shorter ones.

(6) *During regeneration in the adult the normal relationship between internodal length and fibre diameter does not reappear*

The general conclusion on this subject which emerges from the results of the present experiments is that following regeneration the normal linear relationship between internodal distance and fibre diameter does not reappear. As late

as 16 months after interruption of the nerve by crushing the largest fibres present have internodes far shorter than those of fibres of similar diameter in a normal nerve. At this late stage, however, the population of fibres is normal as regards size and myelin thickness, and the conduction rate of the fastest fibres is restored to normal (Sanders & Whitteridge, 1946). In fact, as a generalization, it is possible to say that following regeneration there is no correlation between internode length and diameter. This remarkable result follows, according to the above hypothesis, from the fact that, with one exception to be discussed later, these experiments were made on adult animals in which little or no growth took place during regeneration. The myelin on regenerating fibres apparently at first forms a continuous cylinder, in the animals examined 60 and 90 days after operation considerable stretches of uninterrupted myelin were found. The Schwann cell nuclei have of course multiplied by as much as thirteen times and will not therefore at first be spaced at regular intervals along the new fibres, this continuous column of Schwann cells can hardly produce regular segmentation of the myelin. Nevertheless, as the diameter increases, the myelin breaks into segments, presumably, on our hypothesis, as a result of its surface tension, just as a secondary breaking of myelin occurs during the rapid growth 2-4 weeks after birth (p. 121), or when fibres were artificially increased in diameter by placing in hypotonic solutions (Young, 1945). A simple physical explanation for the segmentation is suggested by the fact that its minimum periodicity resembles that seen in normally developing fibres. However, there is from the start considerable variation in the length of regenerating internodes, as might perhaps be expected under the varying conditions of the Schwann tubes.

There being during regeneration no growth of the limb as a whole, the internodes of the earliest developed fibres do not subsequently become more elongated than those of the fibres which were later in developing their sheaths, and the normal relation between diameter and internodal length does not appear. However, a very striking feature after regeneration in the adult is the wide distribution of internode length for any given diameter, and the significance of this variation is not yet clear. It may perhaps be greater in the earlier stages, suggesting a possible disappearance of some of the earliest nodes, leading to fusion of segments. There are certainly fewer very short internodes after long periods of regeneration.

The one case in which regeneration was studied in a growing animal is consistent with this hypothesis. The difference between the nerve in this case and the nerves produced in adult animals after similar time of regeneration is that the scatter is less and the internodes longer in the young animal. This would be expected if the process of obliteration of nodes mentioned above is complicated by growth, stretching the segments once formed. As nodes are not formed until regeneration has been going on for some time we can understand that the longest internodes are shorter after the regeneration in this growing animal than in a normal adult, since the nerve was interrupted 11 days after birth.

CONCLUSION

Significance of the nodes of Ranvier

The hypothesis that the myelin segment is an enclosed droplet thus fits the observed results throughout. More physical knowledge is undoubtedly required about the factors operating in the maintenance of the nerve fibre, such as the rate of production of axoplasm and myelin, the viscosity of axon and myelin, and the resistance to stretch of the neurilemma, before the theory can rest upon a firm basis. The changes in internodal length which have been observed all suggest, however, that the myelin behaves as a fluid.

Unfortunately this does not tell us what is the significance of myelin segmentation in the life of the animal. The investigations of Sanders & Whitteridge (1946) on conduction velocity during regeneration, which included measurements made on the regenerated nerves here described, show that there is no direct relationship between the spacing of the nodes and conduction velocity. They found that conduction velocity increases with diameter in the regenerated nerves very much as in a normal adult animal, the larger fibres conduct faster than the smaller in spite of the fact that all have the same length of internode. It is important also to remember that the myelin of fibres of the central nervous system shows no nodes, perhaps because it is not enclosed in the neurilemmal tubes found in peripheral nerves (Young, 1945). This difference by itself suggests that nodes are not essential for propagation of nerve impulses.

In view of these facts it is difficult to believe the theory that length of internodal segment is an important factor determining velocity of nerve conduction. If the above hypothesis is correct the segmentation of the myelin of peripheral fibres is due to the presence of an inextensible neurilemmal tube which is not wetted by the myelin. The tube no doubt serves to support the peripheral axons, which are liable to considerable strain by reason of their position in the limb, it seems likely that the segmentation of the myelin is a further factor preventing damage to the fibres. Liquid material would very readily be pushed along a tube, division into a series of elongated droplets makes this much more difficult and may thus protect the fibre against disruption. The evidence at present available suggests that the nodes of Ranvier have thus 'protective' function or possibly that their presence is a physical result of the protective tubes, and without any special adaptive significance. Gaps in the myelin layer, however caused, no doubt profoundly modify the electrical conditions and affect the direction of current flow, but the periodicity of the break does not appear to influence the velocity of conduction and it is therefore possible that their significance is to be found in some aspect of the life of peripheral nerve not primarily connected with impulse transmission.

SUMMARY

1 In the peroneal nerve of adult rabbits there is a linear relationship between internodal length and fibre diameter. Internodes on fibres of 3–18 μ range from 0.40 to 1.50 mm in length.

2 Occasionally internodes are found which are twice the normal length, without variation in the diameter of the fibre. Stretches with short internodes are also found and these are sometimes associated with a reduced diameter over this stretch.

3 In normal development the myelin on its first appearance is divided into segments separated by long nodes, leaving considerable lengths of bare axon.

4 The original segments may temporarily divide into smaller droplets during the period of 2–4 weeks after birth.

5 The length of the internodal segments increases with the length of the whole nerve, and the diameter also increases with age.

6 The number of internodal segments in a stretch of nerve fibre, therefore, remains constant throughout development. The linear relationship between internodal length and diameter found in the adult is a result of the fact that the fibres which will ultimately become the largest are the first to myelinate and their internodes are therefore the more stretched by growth after their formation.

7 In regeneration the myelin at its first appearance is continuous over the surface of the whole fibre and there are no nodes. It then divides into short segments varying from 0.15 mm upwards to 0.7 mm. After long periods of regeneration, therefore, all fibres have similar internodal lengths, although the range of diameters has become normal (3–18 μ).

8 A nerve fibre made to regenerate soon after birth passes through the periods of division into internodes plus the stretching of the nerve by the growth of the limb. Consequently, the internodal length of the segments found is considerably larger than in regenerated nerves of adult animals.

9 All these relations can be understood by the assumption that an internodal segment of myelin is a liquid droplet maintained in the shape of a cylinder by the outward pressure of the axon and the resistance to stretch of the neurilemma.

10 The results, taken with the experiments of Sanders & Whitteridge on the conduction velocity of the same regenerated nerves, show that there is no necessary relation between conduction velocity and internodal length.

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EXPLANATION OF PLATE 1

Fig 1 Short internodes after regeneration A and B, normal fibres of rabbits' peroneal nerve, C and D, fibres from a nerve which had regenerated for 486 days after interruption by crushing. Fibres fixed in formol and stained with osmium tetroxide

Fig 2 Fibres of peroneal nerve of 4 week old rabbit, showing breakage of myelin into droplets and long nodes. Fixed and stained with 1% osmium tetroxide

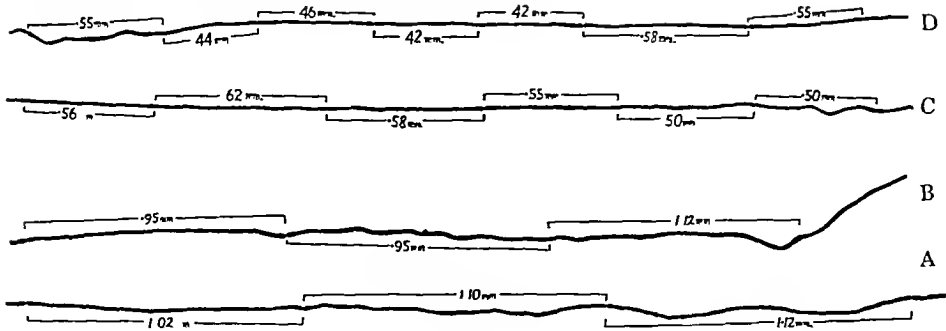


Fig 1

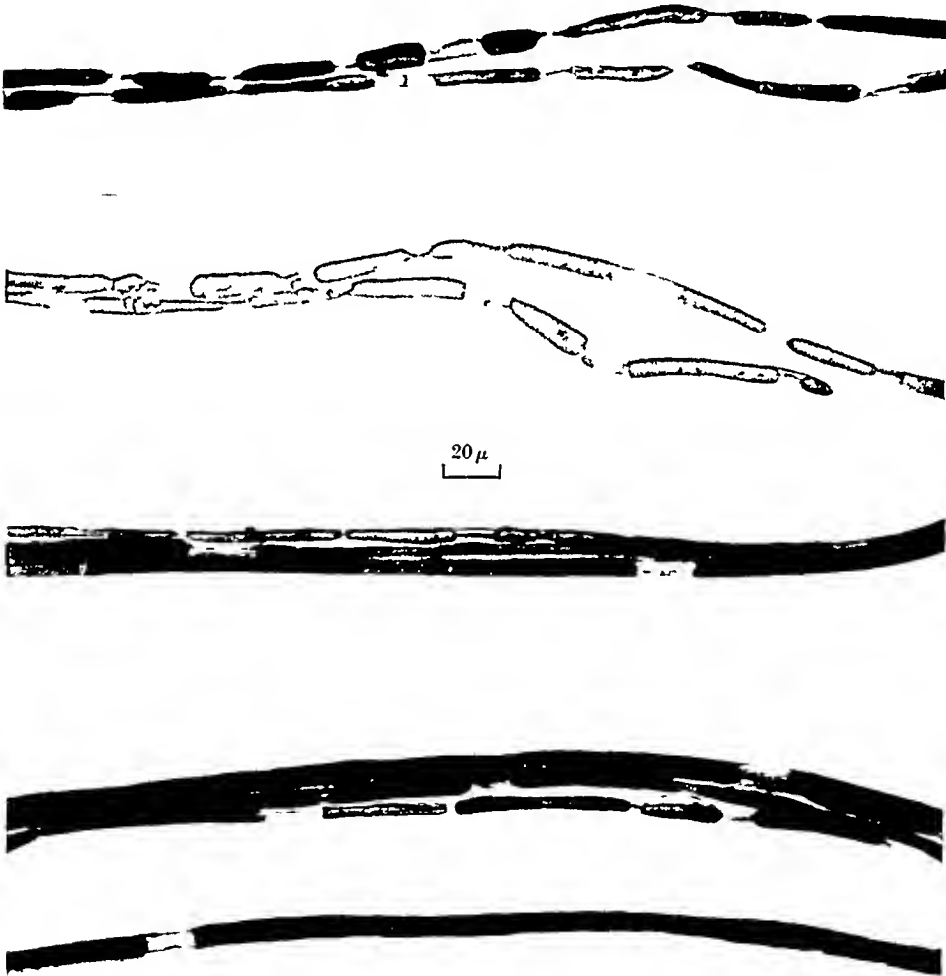


Fig 2

QUANTITATIVE HISTOLOGY OF WALLERIAN DEGENERATION

II NUCLEAR POPULATION IN TWO NERVES OF DIFFERENT FIBRE SPECTRUM

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INTRODUCTION

Abercrombie & Johnson (1946), in Part I of this investigation of the quantitative histology of Wallerian degeneration, found that the number of nuclei in rabbit peroneal and tibial nerves increased to a maximum at about 25 days of degeneration, after which it declined somewhat, though at 100 days it was still five times as great as that of undegenerated nerve. They analysed the nuclear population into various sub-populations, those of endoneurial connective tissue, blood-vessel walls and Schwann tubes, dividing the Schwann tubes into those of large, medium and small calibre. Each sub-population was found to multiply to a different extent during degeneration. Since there were big differences in the amount of multiplication of nuclei of Schwann tubes of different sizes, Abercrombie & Johnson suggested that the multiplication of nuclei in any nerve undergoing degeneration would be found to depend, at least in part, on its fibre spectrum (that is, on the relative numbers of nerve fibres of different sizes which make up the nerve). The present investigation was undertaken, first, in order to discover whether a similar general pattern of rise and fall of nuclear numbers occurs in nerves other than the peroneal and tibial, and secondly, to find out whether there are quantitative differences in the magnitude of changes of numbers of nuclei which could be correlated with differences in the fibre spectra of the nerves. Both problems bear on the wider problem of the mechanism of mitotic stimulation.

In this paper the changes during degeneration in the number of nuclei in the nerve to the medial head of the gastrocnemius muscle (henceforward called the *ngm*) and in the sural nerve of the rabbit are surveyed. The *ngm* and sural nerve were chosen because they both occur in the same region of the thigh, immediately alongside the peroneal and tibial nerves previously studied, but are very different in fibre spectrum, the *ngm* consisting mainly of large fibres, and the sural nerve mainly of small ones. The average fibre diameter of the peroneal and tibial nerves is similar and roughly intermediate between that of the *ngm* and sural nerve. The anterior tibial nerve also was studied, but not in such detail as the *ngm* and the sural nerve. The anterior tibial is the distal branch of the peroneal nerve in the shank, its fibre spectrum is similar to that of the sural nerve but, since it is in a different anatomical region, it gives some

opportunity of discovering whether factors other than the fibre spectrum of a nerve affect its changes of nuclear population during degeneration

The nuclei of the different types of cell have as far as possible been considered separately, as was done by Abercrombie & Johnson. I have distinguished between nuclei within the Schwann tubes (tubal nuclei) and nuclei in the endoneurium. These latter have been analysed into connective tissue nuclei and blood-vessel wall nuclei. The tubal nuclei have not, however, been analysed according to the size of tube in which they were contained.

MATERIAL AND METHOD

The operative procedure was as described by Abercrombie & Johnson (1946). The nerve was cut in the thigh region and allowed to degenerate for the required period, after which the animal was killed and part of the peripheral stump removed and fixed. The centimetre of peripheral stump adjacent to the cut made at operation was not used for counting, since it was likely to have been affected by the trauma of the operation. For undegenerated material the corresponding region of nerves in unoperated rabbits was removed and fixed. Such undegenerated nerves are referred to as 0-day nerves.

The periods of degeneration studied were 5, 10, 25, 50 and 100 days for the *ngm* and sural nerve. The nerves are correspondingly referred to as 5-day, 10-day, etc. In addition, sural nerves at 15 days of degeneration and anterior tibial nerves at 10 and 25 days were taken.

112 nerves from 43 rabbits have been used.

Histological treatment was as described by Abercrombie & Johnson (1946). Longitudinal and transverse sections at 7μ were used. The sural nerve was attached to a small piece of card to prevent distortion during fixation but, since the *ngm* is firmly bound to the tibial nerve throughout the region used, there was no need to support it in this way.

Transverse sections only have been used for counting the nuclear population. To determine the changes in the total population, total counts, i.e. counts of all the nuclei visible in one transverse section, were made. For differential counts, i.e. counts in which the nuclei are analysed into tubal, endoneurial, and blood-vessel wall nuclei, complete transverse sections of the *ngm* were counted, but for the larger and more densely populated sural nerve a number of fields selected at random was counted. When the population of a whole transverse section was to be estimated it was usual to average the counts of three complete sections, selected at random, of each nerve. When fields only were counted, four to ten fields from each of three sections were used.

In the tables which follow, the figure for degenerated nerves has in most cases been expressed as a percentage of the mean number of nuclei in undegenerated nerves. In the analysis of the sural nerve, however, it was found to be impossible to make satisfactory differential counts of undegenerated nerves. But at 5 days of degeneration endoneurial oedema makes the different kinds of nuclei more easily distinguishable. The differential counts of all nerves have, therefore,

been expressed as a percentage of the number of nuclei in 5-day degenerated nerves, at which time the total population has already increased, but only between $1\frac{1}{2}$ and 2 times

Estimations of the statistical significance of differences between means have been made by *t* test, the level of significance used for a conclusion that the samples concerned do not come from the same population being a probability (*P*) that they do so, of 0.01

The necessity for standardizing the nuclear counts for alterations during degeneration in the volume of the whole nerve and in the average length of nuclei was pointed out by Abercrombie & Johnson (1946). The methods used by them, and by Abercrombie (1946), to achieve this standardization have been applied in the present paper

Changes in volume of the nerve produce misleading results only when the data consist of counts of the number of nuclei per microscopical field. When counts of all the nuclei in a complete transverse section of the nerve have been made there is no need to standardize for changes in the volume of the nerve, since changes in volume will not affect the total number of nuclei present. In the differential counts of the sural nerve, the percentages of the various types of nuclei obtained from sample fields have been converted into actual numbers of each of these types of nuclei in a complete transverse section, by applying the percentages to the total counts made separately, of all nuclei in a transverse section of the nerve. Any effect of volume changes on individual fields will thus be allowed for

The method for allowing for changes in nuclear length, used by Abercrombie & Johnson (1946), was used for the sural and anterior tibial nerves, except that the length of the nuclei of every individual nerve was not measured, but the average nuclear length in longitudinal section at each time of degeneration was obtained from a sample of the nerves degenerated for that length of time. Since the small amount of stretching involved in fixing the sural nerve to a piece of card might have affected the length of the nuclei and, therefore, the number of nuclei which appeared in a transverse section, counts of the sural nerve fixed in this way were compared with the counts of nerves which had not been so treated. The difference between these counts was so small, averaging only 6%, that the effect of fixing on cards can be regarded as unimportant in relation to the scale of differences dealt with here

It was found to be impracticable to cut longitudinal sections suitable for measuring nuclear lengths of the very small n.g.m. and, therefore, the method given by Abercrombie (1946) of counting the nuclei in transverse sections of 5μ and 12μ was used

The nuclei of all the nerves studied shorten during early degeneration, and the maximum shortening was found at 10–15 days of degeneration in the sural nerve, and at 25 days in the n.g.m. The nuclei of the n.g.m. shortened most, then those of the peroneal and tibial nerves, and the nuclei of the sural nerve shortened least of all

RESULTS

Changes in total nuclear population

Table 1 shows the mean number of nuclei in a complete transverse section of the n g m and sural nerve at different times of degeneration, corrected for changes in nuclear length, and expressed as a percentage of the mean total number of nuclei in undegenerated nerves. Column 3 shows the figures for the nuclei of the n g m and column 5 those for the nuclei of the sural nerve. The data are shown graphically in Text-fig. 1 along with the data for peroneal and tibial nerves obtained by Abercrombie & Johnson (1946) (henceforward referred to as peroneal-tibial data).

Table 1 *Mean populations of all nuclei of nerves at different times of degeneration, expressed as percentages of the mean populations of undegenerated nerves, with standard errors*

(1) Days of degeneration	N g m		Sural	
	(2) No of nerves	(3) Mean population (%)	(4) No of nerves	(5) Mean population (%)
0	8	100 ± 9	9	100 ± 5
5	8	202 ± 13	9	140 ± 6
10	7	740 ± 30	9	310 ± 22
25	8	1860 ± 97	9	470 ± 21
50	6	1370 ± 60	7	280 ± 10
100	6	1120 ± 36	6	252 ± 13

In all three nerves the population increases quickly to a maximum and thereafter declines, more rapidly at first, less rapidly later. But though the changes in nuclear population of the three kinds of nerves have the same general trend, there are considerable differences in the amounts of multiplication which the nuclei of the different nerves undergo. The nerves with the larger fibres show the greater degree of multiplication. Pl. 1, figs. 1-4, showing degenerated and undegenerated sural nerves and n g m, illustrate the differences.

Taking the n g m or sural nerve separately, the differences between total populations at different times of degeneration are all statistically significant, except for the differences between the populations at 50 and 100 days in both nerves. At 25, 50 and 100 days of degeneration, but not at 5 and 10 days, the percentage increase of the nuclear population of each kind of nerve is significantly different from that of either of the other kinds.

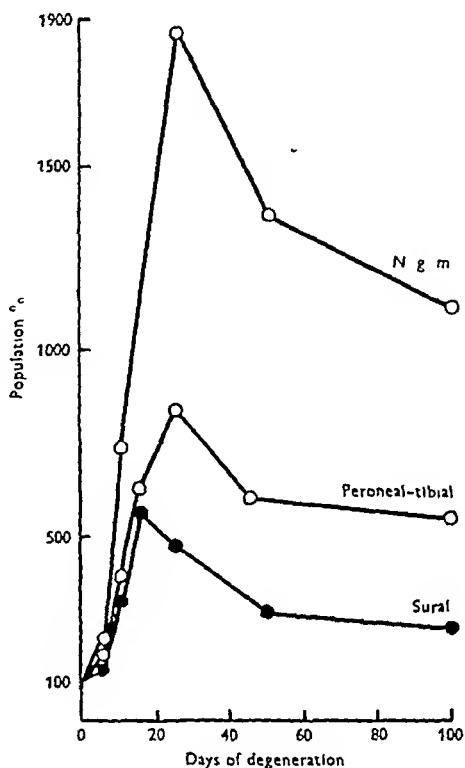
The main nuclear estimations were made at 0, 5, 10, 25, 50 and 100 days of degeneration and of these the highest for all nerves were those obtained at 25 days of degeneration. Since, however, measurements have been made at only one time between 10 and 50 days, it is possible that the true peak of population is earlier or later than 25 days. In supplementary experiments it has been shown for the sural nerve that in fact it is earlier. Four sural nerves of 15 days of degeneration, together with the corresponding nerves from the other side of the animals (contralateral controls) of 25 days of degeneration, were prepared. The populations of the nerves degenerated for 15 days were

respectively 118, 126, 122 and 121 % of the populations of their 25-day contralateral controls. The difference between the populations at 15 and 25 days is statistically significant. Further investigation would probably show that the population peaks of the n g m and peroneal-tibial nerves are not exactly at 25 days of degeneration, though it is improbable that the maximum populations have been seriously underestimated.

At its maximum (at 15 days of degeneration) the nuclear population of the sural nerve has increased 6 times, while that of the n g m has at its maximum (at 25 days) increased 19 times. In comparison the peroneal-tibial population has increased 8 times by 25 days. The maximum recorded n g m population increase is therefore more than 3 times that of the sural nerve and more than twice that of the peroneal-tibial nerves.

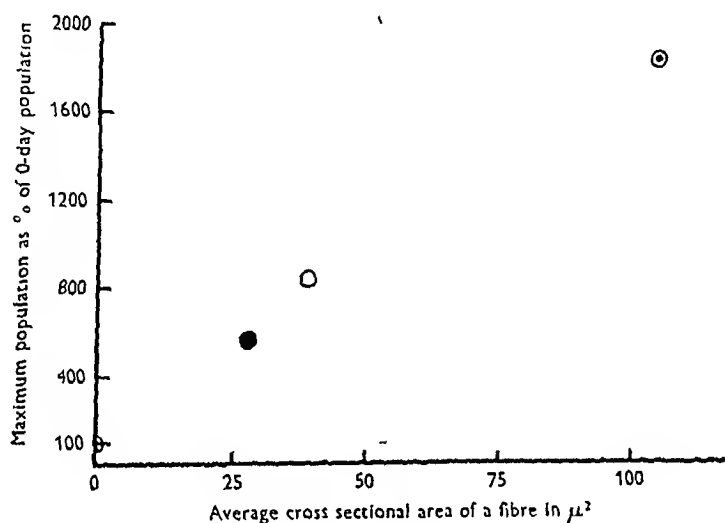
By 100 days of degeneration the nuclear population of the n g m has declined to 60 % of the peak population, that of the sural nerve to 44 % and that of the peroneal-tibial nerves to 64 %, the decline being most rapid immediately after the presumed peak of population has been reached.

An undegenerated sural nerve averages 211 nuclei per transverse section of 7μ thickness and an undegenerated n g m averages 41 nuclei, the cross sectional areas of the two kinds of nerve being roughly similar. Since the n g m has a higher rate of nuclear multiplication than the sural nerve the populations of the two nerves become more and more alike numerically as degeneration proceeds. Thus, at 25 days of degeneration, the sural nerve averages 990 nuclei per transverse section and the n g m 770. In 1 cu mm of undegenerated nerve there are roughly 90,000 nuclei in the case of the sural nerve, 27,000 in the n g m and 43,000 in the peroneal-tibial nerves. By 25 days of degeneration the differences between the number of nuclei in the different nerves is much less, the sural nerve population being 440,000, the n g m 450,000 and the peroneal-tibial 330,000 nuclei per cu mm. These figures refer of course to fixed material.



Text fig 1 Mean populations of nuclei of three different nerves at different times of degeneration, expressed as percentages of those of undegenerated nerves. ● Sural nerves, ○ peroneal tibial nerves, ○ n g m

Abercrombie & Johnson (1946) suggested that the changes in nuclear population during degeneration of any nerve are likely to depend at least in part on its fibre spectrum. The above data show such a relationship. One aspect of the fibre spectrum which we can examine is the average cross-sectional area of a myelinated fibre of the nerve. In Text-fig 2 the nuclear population of each kind of nerve, when it is at its maximum (as far as my data reveal the maximum), is plotted against an estimate of the average cross-sectional area of a fibre. The figures for the n g m and sural nerve average fibre areas were very kindly supplied by Dr G Causey, and those for the peroneal-tibial nerves were calculated from data published by Gutmann & Saunders (1943).



Text fig 2 Maximum populations of nuclei of four different nerves expressed as percentages of the nuclear populations in these nerves when undegenerated, plotted against the average cross sectional area of a nerve fibre of the respective nerves. ● N g m, ○ peroneal tibial nerves, ● sural nerves, ○ anterior mesenteric nerves

The histological method used by Dr Causey to obtain the average fibre areas was that used by Aitken, Sharman & Young (1947). It involves a shrinkage of the living fibre diameter probably not exceeding 10%. In measuring the diameters, the nerve fibres were divided into groups which differed from each other in diameter by 2μ . The number of fibres in each group was then multiplied by a figure for the cross-sectional fibre area calculated from the median fibre diameter of the group. The weighted mean of the average fibre areas so obtained for each group is the average fibre area of the whole nerve.

The points on the graph in Text-fig 2 represent the maximum percentage increase of the nuclear populations of the n g m, the peroneal-tibial and sural nerves, and since Joseph (1947) showed that the nuclei of an unmyelinated nerve do not increase in number at all during degeneration, the zero point can be put in, representing the anterior mesenteric nerve which he used. As a rough approximation it appears that the amount of nuclear proliferation in the nerves is directly proportional to the average cross-sectional fibre area.

The relationship which thus appears to hold between the average fibre area and the amount of proliferation in a nerve has been further checked on the anterior tibial nerve. If the relationship really exists, then the anterior tibial nerve should show changes in population more like those in the sural nerve than those in the peroneal from which it arises, since the average fibre area of the anterior tibial nerve (Sanders & Young, 1944) is very similar to that of the sural nerve. This proved to be the case. At 25 days of degeneration six anterior tibial nerves had a nuclear population which was 146 % of the nuclear population of six other anterior tibial nerves at 10 days of degeneration. The comparable figure for sural nerves is 150 %. The g m and peroneal-tibial nerves, with their very different fibre spectra, showed increases in nuclear population of 260 and 215 % respectively.

Differential counts of nuclear multiplication

In order to discover to what extent each of the nuclear categories present was responsible for the differences in the amount of proliferation in the different nerves, the populations have been analysed into tubal nuclei (i.e. those nuclei which lie within the Schwann tubes), endoneurial connective tissue nuclei, and blood-vessel wall nuclei.

The difficulties involved in the recognition of the different kinds of nuclei in the undegenerated sural nerve, which has a large number of small fibres, were found to be insuperable. Therefore, in considering these differential counts, the figure for a nerve of 5 days of degeneration has been used as a standard from which to calculate the relative amount of nuclear increase.

The figures for the populations of different nuclear types have been standardized for changes in nuclear length during degeneration, though for this standardization the average length of all the nuclei in the nerve was used and not the average length of each individual type.

The nuclei of the sural nerve were not counted differentially at 15 days of degeneration, but in the light of the total counts at this time, it should be remembered that the peak population of each of the different categories of its nuclei is likely to lie nearer to 15 days than to 25 days.

Changes in numbers of tubal nuclei

The population of tubal nuclei in both kinds of nerve shows, like the population of all nuclei, a rise to a maximum followed by a decline. As with the total population, the g m tubal nuclei show a much greater increase than those of the sural. They reach 17 times the 5-day population at 25 days. The peroneal-tibial tubal nuclei increase 8 times and those of the sural nerve only 4 times.

The third columns in Tables 2 and 3 show the changes in tubal populations with time of degeneration.

Taking each kind of nerve separately, at each time of degeneration studied the tubal nuclear population is significantly different from the tubal nuclear population at either the preceding or the following times of degeneration.

After 10 days of degeneration the tubal nuclear population of any of the three kinds of nerve is significantly different from that of either of the others

Table 2 *Nervus gastrocnemii medialis* Mean populations of various nuclei of nerves at different times of degeneration, expressed as percentages of the mean populations of 5-day degenerated nerves, with standard errors

(1) Days of degeneration	(2) No of nerves	(3) Tubal nuclei	(4) Endoneurial nuclei	(5) Blood vessel wall nuclei
0	8	47±5	53±5	45±5
5	8	100±5	100±8	100±7
10	7	606±18	188±12	107±9
25	8	1680±96	336±18	193±22
50	6	1220±67	260±12	143±17

Table 3 *Sural nerve* Mean populations of various nuclei of nerves at different times of degeneration, expressed as percentages of the mean populations of 5-day degenerated nerves, with standard errors

(1) Days of degeneration	(2) No of nerves	(3) Tubal nuclei	(4) Endoneurial nuclei	(5) Blood vessel wall nuclei
5	8	100±6	100±6	100±13
10	7	286±16	158±9	109±8
25	8	435±29	230±17	120±10
50	6	254±17	144±9	107±8

Changes in numbers of endoneurial connective tissue nuclei

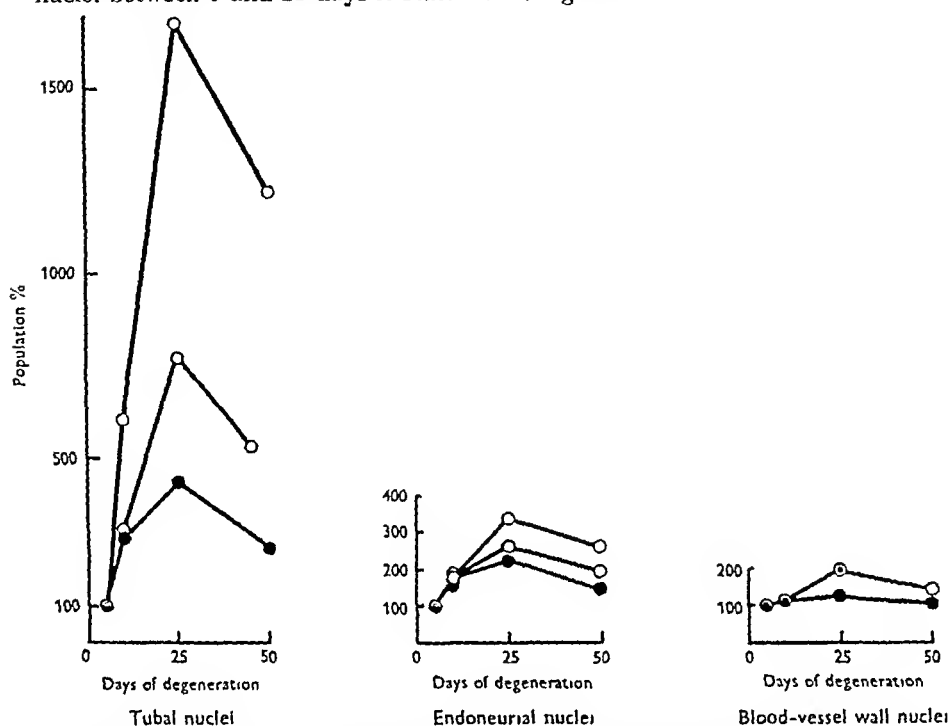
The numbers of endoneurial nuclei are shown in Tables 2 and 3, column 4, expressed as a percentage of the mean population of nuclei at 5 days of degeneration. It will be seen from Tables 2 and 3 and Text-fig 3 that the endoneurial nuclei increase much less than the tubal nuclei, but there is still a peak at 25 days of degeneration. There is a difference in the amounts by which the endoneurial nuclei of the different nerves increase. In the n g m at 25 days of degeneration the endoneurial nuclei are $3\frac{1}{2}$ times as numerous as at 5 days, in the sural nerve twice as numerous, and in the peroneal-tibial $2\frac{1}{2}$ times as numerous. In each case the 25-day figure is significantly different from the 5-day figure.

Changes in numbers of blood-vessel wall nuclei

It is possible to count blood-vessel wall nuclei (endothelial and smooth muscle nuclei) in undegenerated nerves and so the nuclear populations in this section are expressed as percentages of the 0-day population of nuclei. In the fifth column of Tables 2 and 3, however, and in Text-fig 3 the nuclear populations of the blood-vessel walls have been expressed as a percentage of the population at 5 days, so that they can be compared directly with the figures for other nuclei.

At 25 days of degeneration the blood-vessel wall nuclei of the n g m have increased to 470% of the undegenerated population, the peroneal-tibial to 146% and the sural to 145%. At this time the increase in the blood-vessel wall nuclear population of the n g m is significantly different from that in the

sural and peroneal-tibial nerve populations, though the increase of these latter are not significantly different from each other. The increase in blood-vessel wall nuclei between 0 and 25 days is statistically significant in each of the nerves



Text fig 3 Mean population of tubal nuclei, endoneurial nuclei and blood vessel wall nuclei of three different nerves at different times of degeneration expressed as percentages of the respective nuclear populations in 5 day degenerated nerves ● Sural nerves ○ peroneal tibial nerves ○ n g m

Synthesis of differential counts

The changes of populations of the different kinds of nuclei of the n g m and sural nerve are brought together in Table 4, which gives the composition by cell type of a representative sample of 100 nuclei in 5-day degenerated nerves, and shows the number of nuclei which such a sample produces by multiplication as degeneration proceeds

Table 4 Changes which occur in the tubal, endoneurial and blood-vessel wall nuclear populations in a region of nerve which at 5 days of degeneration contains 100 nuclei

Days of generation	N g m				Sural			
	Total nuclei	Tubal nuclei	Endoneurial nuclei	Blood vessel wall nuclei	Total nuclei	Tubal nuclei	Endoneurial nuclei	Blood vessel wall nuclei
5	100	45	41	14	100	57	36	7
10	366	274	77	15	227	162	57	8
25	920	755	138	27	340	248	83	9
50	677	550	107	20	206	146	52	8

DISCUSSION

It now seems that the general pattern of change in nuclear population found by Abercrombie & Johnson (1946) is by no means peculiar to the peroneal and tibial nerves, but that other myelinated nerves of very different fibre size, namely, the nervus gastrocnemii medialis (n g m) and sural nerve, show this rise and fall of nuclear population with approximately the same time relations.

Although the nerves studied are similar in the general pattern of their multiplication, the amounts of increase are very different, and Abercrombie & Johnson's suggestion that the extent of the population increase in any nerve will depend at least in part on its fibre spectrum is amply borne out by these figures for a variety of nerves. The present work, together with that of Joseph (1948), indicates a very close correlation between the extent of the increase in size of the nuclear population of a nerve during Wallerian degeneration and the characteristic of the fibre spectrum which we use here, the average cross-sectional area of a myelinated fibre of the nerve. The larger the average fibre area, the greater is the increase in nuclear population relative to the initial population, the two variables being roughly linearly related.

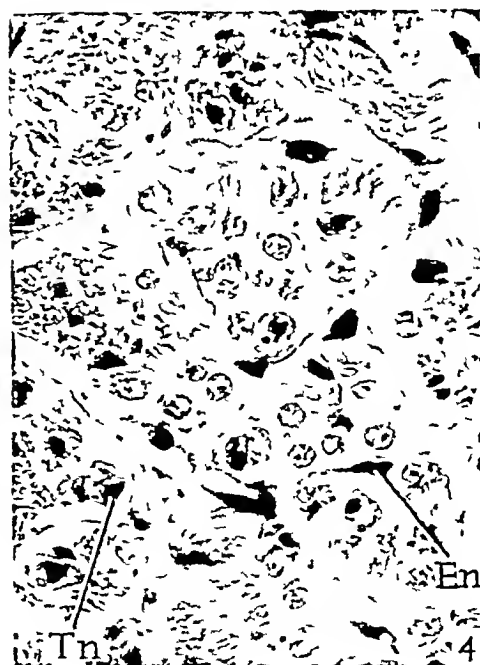
The cardinal problem which initiated the present studies of nuclear population is the causation of mitosis. Abercrombie & Johnson (1946) postulated a diffusing activator of mitosis produced by autolysis of nerve fibres. The results recorded here give no conclusive evidence for or against this hypothesis, though the fact that, in different kinds of nerve, the greater the nuclear multiplication is in the tubes the greater it is in the endoneurium is at least consistent with the view that both tubal and endoneurial cells are responding to the same activator. What the present work does demonstrate is a fundamental relation between nuclear multiplication and a quantitative characteristic of degenerating nerve fibre material. The discovery of this relationship clearly opens the way to further investigation of the mechanisms of mitotic stimulation involved.

SUMMARY

1 The changes in nuclear populations during Wallerian degeneration for different periods of two rabbit nerves of differing average fibre size, the nervus gastrocnemii medialis (n g m) (large fibres) and the sural nerve (small fibres) are recorded.

2 The total nuclear populations of the nerves show changes similar to those established by Abercrombie & Johnson (1946) for the peroneal and tibial nerves, the growth curve showing a rise to a maximum population, followed by a fall which does not, however, reduce the population to that of undegenerated nerve.

3 After 25 days of degeneration the nuclear population of the n g m had increased to 19 times its value in the undegenerated nerve, while the sural nerve had only increased to 5 times its value in the undegenerated nerve. By 100 days of degeneration the nuclear population of the n g m had fallen to



11 times the population in the undegenerated nerve. In the same time the population of the sural nerve had fallen to $2\frac{1}{2}$ times the population in the undegenerated nerve.

4 The maximum amounts of nuclear multiplication in the different nerves, including also the peroneal and tibial nerves (Abercrombie & Johnson, 1946) and the anterior mesenteric nerve (Joseph, 1947), have been correlated with the average cross-sectional area of a nerve fibre of these nerves. There exists a roughly linear relation between the two variables.

5 The nuclei of the nerves have been analysed into those of the Schwann tubes, of the endoneurium and of the blood-vessel walls. Each category shows a rise followed by a slight fall in population. The extent of the rise is related to the average fibre size. The nuclei inside the Schwann tubes have, by 25 days of degeneration, increased to 17 times their population at 5 days in the n g m and to $4\frac{1}{2}$ times in the sural nerve. Those of the endoneurium have increased to $3\frac{1}{2}$ times their population at 5 days in the n g m and to $2\frac{1}{2}$ times in the sural nerve. Those of the blood-vessel walls have increased to twice the population at 5 days in the n g m and to 1.2 times in the sural nerve.

I should like to thank Mr M. Abercrombie for supervising the conduct of this work, and Profs G. R. de Beer and J. Z. Young for criticizing the manuscript. The work was done while holding a studentship from the Medical Research Council.

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EXPLANATION OF PLATE 1

Preparations of rabbit nerves fixed in Susa, stained in Heidenhain's haematoxylin and light green, sectioned transversely at 7μ . T n tubal nucleus E n endoneurial nucleus Magnification $\times 640$.

- Fig. 1 Undegenerated ncrvus gastrocnemii medialis
 Fig. 2 Peripheral stump of nervus gastrocnemii medialis degenerated for 100 days
 Fig. 3 Undegenerated sural nerve
 Fig. 4 Peripheral stump of sural nerve degenerated for 100 days

CHANGES IN NUCLEAR POPULATION FOLLOWING TWENTY-ONE DAYS' DEGENERATION IN A NERVE CONSISTING OF SMALL MYELINATED FIBRES

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INTRODUCTION

There is a remarkable difference between the eightfold increase in nuclear population at the end of 25 days' degeneration in a typical somatic nerve as shown by Abercrombie & Johnson (1946), and the absence of cell multiplication after a similar period in a non-myelinated nerve as shown by Joseph (1947). It was thought that an investigation into nuclear changes which occur following degeneration in a nerve consisting of small fibres might help to elucidate the causes of this difference and also shed some light on the nature of the stimulus or stimuli which cause cell multiplication. This paper deals with a study of the nuclear changes in a nerve consisting almost entirely of fibres less than 6μ in diameter. Thomas (1948), in an independent investigation, has studied the changes in nerves consisting of fibres of larger size.

MATERIAL AND METHOD

The nerve chosen was the greater splanchnic nerve of the rabbit. Simpson & Young (1945) showed that only 4% of the fibres of this nerve are larger than 6μ in diameter. The left nerve was cut with fine sharp scissors just where it emerges below the diaphragm, as it lies beside the abdominal aorta. The intact right nerve was used for comparison. An attempt was made to carry out a similar operation on the right side, but this was found to be impossible owing to the difficulty of avoiding a fatal pneumothorax. In addition, the liver tore very readily in attempting to expose the site of operation and caused very troublesome bleeding. Since, therefore, all the interruptions were made on the left splanchnic nerve it was necessary to investigate any possible difference between the right and left nerves in normal rabbits. In eight experimental rabbits 21 days after operation, both right and left nerves were removed, and each nerve was divided into a proximal and distal piece. Similarly, in 7 normal rabbits four specimens were obtained, two from each side. Each specimen was 1-2 cm long. They were fixed in Bodian's fluid containing 15 ml formaldehyde, 5 ml acetic acid and 80 ml of 80% methyl alcohol. Transverse sections at 5μ of each end of all four specimens were cut and two slides of each were prepared. One slide was stained for axons by Bodian's method, and one with haematoxylin and eosin. The absence of normal axons in the sections of the Bodian-stained slide confirmed that the nerve had been completely interrupted. Photographs at a magnification of $\times 350$ of sections stained with haematoxylin and eosin from two different levels from each side of each rabbit were used for counting nuclei.

RESULTS

Table 1 shows the number of nuclei at two levels in the right and left nerves of 7 normal rabbits. Frequently, the greater splanchnic nerve in the rabbit consists of more than one nerve bundle (Pl 1, fig 1). Where this occurred, the nuclei in all the bundles were counted and added together. The nuclei of all cells within the perineurium were counted. The variability which is obvious in these nuclear counts may be due to differences between rabbits, between the different sides of the same rabbit, between the different levels of the same nerve, or may be due to these factors acting together. It is important that one should be able to isolate the variability due to these different factors and reach a decision as to the relative significance of each. The method for reaching this decision is the analysis of variance discussed by Fisher (1946). The Table resulting from this analysis is given in an Appendix (Table 3), and shows that

Table 1

Serial number of rabbit	Level	Number of nuclei per transverse section at 5 μ	
		Right (normal)	Left (normal)
184	Proximal	295	452
	Distal	430	540
337	Proximal	279	249
	Distal	266	337
338	Proximal	421	458
	Distal	508	379
348	Proximal	271	260
	Distal	325	303
350	Proximal	324	308
	Distal	318	366
380	Proximal	304	258
	Distal	272	266
422	Proximal	335	257
	Distal	357	213

variations due to differences between the two sides and different levels are not significant at the 5 % level. On the other hand, the variation between individual rabbits is clearly significant, as might be expected.

Table 2 shows the number of nuclei at two levels in the right and left nerves in 8 rabbits, in which the left nerve was cut and allowed to degenerate for 21 days (Pl 1, fig 2). The two levels at which the nuclear counts were made were 5 mm. or more from the site of the cut.

The greater splanchnic nerve consists largely of preganglionic fibres. In many of the nerves ganglion cells occur somewhere along their course. Where they occur distal to the cut the axons of these cells do not degenerate. This results in some uninterrupted fibres being sometimes found in the distal part of the cut nerve. There are never more than a few of these fibres and their presence cannot influence the results.

Abercrombie (1946) showed that the length of nucleus has to be considered in comparing the number of nuclei in the transverse sections of two specimens.

Consequently, two normal nerves were cut in longitudinal section at 7μ and stained with haematoxylin and eosin. Photographs of suitable sections were taken at a magnification of $\times 500$ and the length of 300 nuclei in each nerve was measured. The mean length was 12.60μ in one and 12.92μ in the other. Two degenerated specimens were treated in the same way. The mean nuclear length was 12.71μ in one and 13.91μ in the other. The difference in nuclear length between the normal and degenerated nerves is so small that it can be ignored.

The nuclear counts in Table 2 were subjected to an analysis of variance in the same way as were the counts in the normal rabbits. The Table resulting from the analysis is given in an appendix (Table 4) and shows that, as before, there is no significant variation at different levels. The variation between

Table 2

Serial number of rabbit	Level	Number of nuclei per transverse section at 5μ	
		Right (normal)	Left (after 21 days' degeneration)
128	Proximal	369	547
	Distal	435	627
129	Proximal	305	351
	Distal	265	451
174	Proximal	320	361
	Distal	417	334
175	Proximal	312	600
	Distal	397	600
176	Proximal	234	362
	Distal	244	295
351	Proximal	444	447
	Distal	413	618
383	Proximal	430	665
	Distal	493	580
384	Proximal	242	321
	Distal	272	325

rabbits is still significant, as one might expect. In estimating the variation between the sides the effect caused by differences between rabbits is taken out, and in the experimental animals, unlike the normal animals, the remaining variation between the sides is highly significant. Consequently, it can be concluded that there takes place a definite increase in nuclei after 21 days' degeneration in the greater splanchnic nerve of the rabbit.

In order to obtain some estimation of the extent of this increase the mean percentage difference in the number of nuclei between the right nerve and the left nerve of the 8 experimental rabbits was calculated and found to be 34%.

It was thought that some attempt should be made to determine what types of nuclei had increased in number. It is assumed that the structure of a nerve bundle of a myelinated nerve consisting of small fibres is similar to that of any other myelinated nerve. Thus the nuclei seen in the transverse section of the greater splanchnic nerve can belong to Schwann cells, connective tissue cells,

macrophages or cells associated with blood vessels, endothelial and smooth muscle cells. Of these cells only the Schwann cell is inside the neurilemma. Careful examination of the nuclei in the preparations used for counting failed to show which cells had increased in number. There is usually an obvious increase in the number of blood vessels which results in an increase of blood vessel nuclei from about 5 to 25, but could not account for an increase of 34% in the total nuclei. The increase is therefore due, to a large extent, to an increase in Schwann cells, fibroblasts or macrophages, or all of these.

Abercrombie & Johnson (1946), when studying the nuclei in the sciatic nerve of the rabbit after 25 days' regeneration, divided them into two groups, intratubal and extratubal, and showed that the former multiplied thirteen times and the latter four times. Such a classification in a nerve consisting of small myelinated fibres is impossible since after 21 days' degeneration it is too difficult to determine whether a nucleus is inside or outside the nerve fibre tube. Moreover, since macrophages enter the neurilemmal tubes, especially the large ones, a classification of this kind does not indicate precisely the increase in each type of cell.

In specimens of the greater splanchnic nerve following 6-12 days' degeneration, it is possible to distinguish two types of nuclei, apart from the blood-vessel nuclei, and further study of these nerves may indicate more clearly the changes in number of each type of nucleus.

DISCUSSION

Although Langley (1909), Langley & Orbeli (1911), Ranson & Billingsley (1918), and others, studied degeneration in preganglionic nerves of the sympathetic nervous system, nerves similar to the greater splanchnic nerve of the rabbit in fibre size, they made no special observations about the changes in nuclear population in these nerves. The figures of Table 2 show that there is a striking difference between the increase in nuclei during degeneration in a nerve consisting of small myelinated fibres and one consisting of large fibres. The increase in nuclei in the latter type of nerve is due almost entirely to an increase in macrophages and Schwann cells. Observations regarding macrophages in relation to degenerating small myelinated fibres were made by Weddell & Glees (1941), who showed that there were no cells containing particles in the region of these small fibres following vital dye injections.

When describing degeneration in small myelinated fibres, Nageotte (1932) suggested that the axon and myelin autolyse and 'simply disappear'. He stated that the Schwann cell has the power of phagocytosis in these nerves. If, however, the space within the neurilemma is large enough to receive immigrant cells, then macrophages enter the sheath and phagocytose the debris within it, and in these circumstances the Schwann cells multiply and line the neurilemmal sheath but do not act as phagocytes. Cajal (1928) maintained more definitely that the phagocytes are derived from outside the neurilemmal sheath of the nerve fibre, and that the Schwann cells, although they may have some effect on

the degeneration of the myelin and axon, do not participate in the removal of the products of degeneration. That there is an increase of only 31% in the nuclei of the greater splanchnic nerve after 21 days' degeneration suggests that only a small number of macrophages is required to deal with the degenerated myelin and axon and also that there is practically no increase in Schwann cells.

The size of the neurilemmal tube is therefore evidently a factor in determining the increase in the Schwann cells. Cajal (1928) pointed out that the multiplication of Schwann cells, which begins about the fourth day of degeneration and ends in the third week, is closely related to the shrinking away of the myelin sheath from the Schwann cells. This shrinking results in increased space within the nerve fibre so that the Schwann cell can enlarge and divide. Thus there is probably a relationship between the size of the nerve fibre and the multiplication of Schwann cells, the smaller the nerve fibre the smaller the increase. The different results obtained following degeneration in nerves of different sizes support this.

In order to explain the multiplication of the Schwann cells in the peripheral stump of a degenerated nerve some writers have maintained that this increase is connected with preparation of the peripheral stump for regeneration (Howell & Huber, 1892, Kirk & Lewis, 1917). In the spinal cord, where multiplication of cells did not take place following degeneration, regeneration apparently did not occur (Halliburton, 1907). Regeneration, however, readily takes place both in non-myelinated nerve where there is practically no increase in nuclei after degeneration, and in the greater splanchnic nerve where there is an increase of the order of 34%. It is unlikely that proliferation of the Schwann cells in degenerating nerve fibres plays as important a role in regeneration as earlier writers suggest.

It can be seen from the results of this paper, and those of Abercrombie & Johnson (1946) and Thomas (1948), that there is a considerable difference in the increase in nuclear population at the end of similar periods of degeneration in different peripheral nerves. If it is assumed that the basic structure of the nerve fibres is the same, the main difference between them is the size of the nerve fibres. The results show that the larger the nerve fibres the greater is the increase in the number of nuclei. This difference is almost certainly associated with the greater space within the sheath following degeneration and an increased quantity of the products of degeneration, especially those of the myelin sheath, in large fibres.

SUMMARY

1 In the greater splanchnic nerve of the rabbit, consisting of myelinated fibres of which about 96% are 6μ in diameter or smaller, the number of nuclei in a transverse section 5μ thick shows no significant difference between the right and left nerves and between two levels in the same nerve. There is a significant difference between the nerves of different rabbits.

2 After 21 days' degeneration in this nerve there is a significant increase in the number of nuclei in a transverse section 5μ thick amounting to 31%.

which, it is suggested, is due to a slight increase in all the cells, Schwann cells, macrophages and those of the blood vessels

3 This is compared with the increase seen in nerves consisting of myelinated fibres of greater size, for example, the nerve to the medial head of gastrocnemius which shows a fourteenfold increase, the sciatic nerve which shows an eightfold increase, and the sural nerve which shows a fivefold increase, and a non-myelinated nerve such as the anterior mesenteric nerve which does not appear to show a significant increase

4 The reasons for the increase in nuclei in Wallerian degeneration are discussed and it is suggested that the larger the diameter of the nerve fibres and the greater the quantity of the products of degeneration, the greater is the increase

I wish to thank Prof J Z Young for advice and for reading and criticizing the manuscript, Mr G Hyde for technical assistance, and Mr D Sholl for advice on statistics

APPENDIX

Table 3

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio	Probability level
Sides (S)	54 3214	1	54 3214	—	—
Main effects Rabbits (R)	126833 8571	6	21138 9761	—	—
Levels (L)	6572 8929	1	6572 8929	—	—
S × R	33009 4286	6	5501 5714	—	—
Interactions S × L	150 8928	1	150 8928	—	—
L × R	10754 8571	6	1792 4761	—	—
S × P × L	10823 8572	6	1803 9762	—	—
Total	188200 1071	27			

Since none of the interactions is significant one may pool and then we have

Sides	54 3214	1	54 3214	0 019	N S
Main effects Rabbits	126833 8571	6	21138 9761	7 337	<0 001
Levels	6572 8929	1	6572 8929	2 281	N S
Residual	54739 0357	19	2881 0018	—	—
Total	188200 1071	27			

Table 4

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio	Probability level
Sides (S)	111864 5	1	111864 5	—	—
Main effects Rabbits (R)	286296 0	7	40899 4	—	—
Levels (L)	6498 0	1	6498 0	—	—
S × R	45631 0	7	6518 7	—	—
Interactions S × L	338 0	1	338 0	—	—
L × R	8884 5	7	1269 2	—	—
S × R × L	27589 5	7	3941 4	—	—
Total	487101 5	31			

Since the interactions are non significant they may be pooled

Sides	111864 5	1	111864 5	29 85	<0 001
Main effects Rabbits	286296 0	7	40899 4	10 91	<0 001
Levels	6498 0	1	6498 0	1 73	N S
Residual	82443	22	3747 4	—	—
Total	487101 5	31			

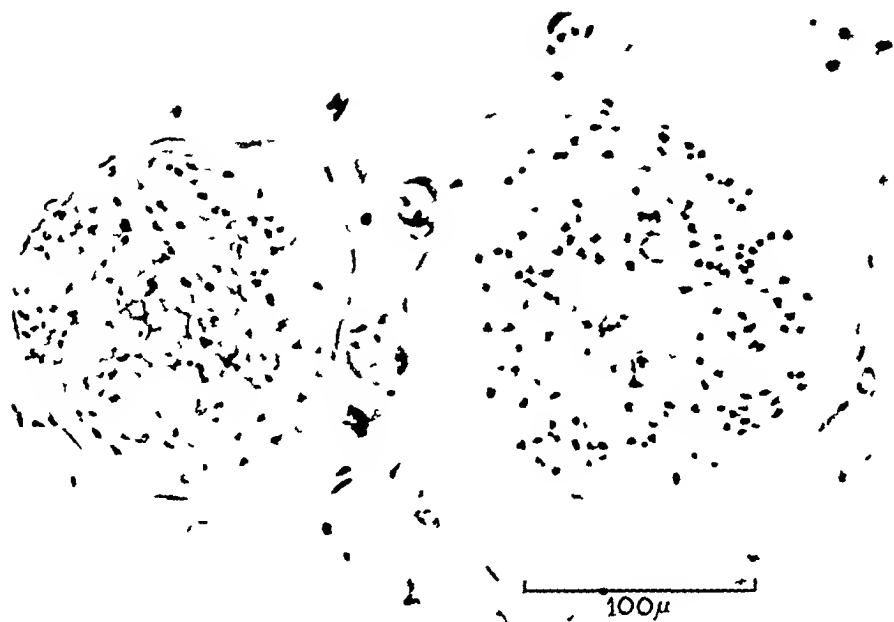
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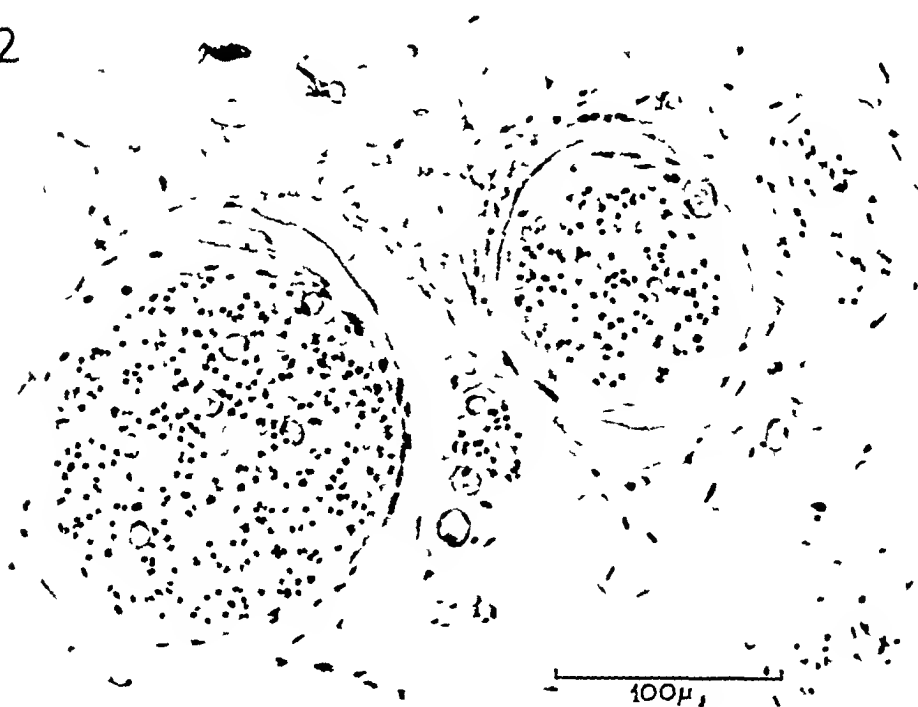
EXPLANATION OF PLATE

- Fig 1 Transverse section of normal greater splanchnic nerve of rabbit Sections 5μ thick Stained haematoxylin and eosin (Rabbit 129)
- Fig 2 Transverse section of greater splanchnic nerve of rabbit distal to cut made 21 days previously Sections 5μ thick Stained haematoxylin and eosin (Rabbit 175)

1



2



THE CONNEXIONS OF THE SPINAL SUB-ARACHNOID SPACE WITH THE LYMPHATIC SYSTEM

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The first clear evidence of a connexion between the cranial sub-arachnoid space and the cervical lymphatic system was provided by Schwalbe (1869), and the existence of such a connexion is now generally accepted although detailed knowledge of the precise pathway is still incomplete. In general, it is believed that the route is from the sub-arachnoid space along the perineural spaces of the olfactory nerves to the tissue spaces of the nasal mucosa and thence, via fine lymph vessels, to the deep cervical lymph nodes.

The normal direction of flow in this sub-arachnoid-lymphatic communication is moreover not yet established. Thus, whilst the cervical lymph nodes can be filled from the cranial sub-arachnoid space under physiological pressures suggesting a centrifugal flow (Quincke, 1872, Weed, 1914, Ivanow, 1927, Galkin, 1930) substances introduced into the nose may later be demonstrated in the sub arachnoid space (Le Gros Clark, 1929). Yoffey & Drinker (1939) discuss the factors involved in determining the direction of flow, but the significance of such factors under physiological conditions is difficult to assess.

That the spinal sub-arachnoid space has a similar connexion with the lymph vessels and nodes of the thorax, abdomen and pelvis has been amply demonstrated by Speransky and his co-workers in a series of papers which have appeared since 1927.

Review of Literature

Schwalbe (1869) introduced Berlin blue under constant but unspecified pressure into the cranial sub-arachnoid space of rabbits and dogs recently killed by exsanguination. As a uniform result he obtained filling of the lymph vessels and nodes of the neck and claimed that the latter were filled through vessels issuing from the jugular foramen in the base of the skull and forming a plexus on the anterior cervical muscles. In one case lymph nodes became injected along the entire length of the vertebral column, and Schwalbe took this to indicate that the spinal sub-arachnoid space had a close connexion with the lymphatic system.

Quincke (1872), using only dogs, injected 1 c.c. of cinnabar into the spinal sub arachnoid space through a laminectomy opening in the upper lumbar region. The animals survived from 2 to 4 days. Quincke concluded that

(1) A part of the cerebro-spinal fluid leaves the sub arachnoid space along the nerves.

(2) There is a sac of arachnoid around the issuing nerve roots in which cinnabar collects and is not usually carried further except in the optic nerve.

(3) The cerebro-spinal fluid passes into the cervical and sub-maxillary lymph nodes

As a serious contribution to this problem, the much quoted injection work of Key & Retzius (1875) is of little value on account of the high pressure used (60 mm of mercury)

The same objection applies even more forcibly to the work of Spina (1900-1), for all his injections, with one exception, were carried out at pressures from 120 to 400 mm of mercury

Sub-arachnoid injection of a 1% solution of trypan blue was used by Goldmann (1913), who effected this both through a parietal trephine hole in the skulls of dogs and a type of lumbar puncture into the spinal sub-arachnoid space of rabbits. A small amount of cerebro-spinal fluid was first withdrawn, and the quantity of trypan blue injected varied from 1.0 to 2.5 c.c. Only when the latter volume was injected could staining of the deep cervical nodes be seen. Once again one must comment upon failure to control the pressure of injection.

Weed (1914) introduced into the sub-arachnoid space of the dog an isotonic mixture of potassium ferrocyanide and ferric ammonium citrate, and demonstrated granules of Prussian blue in the cervical lymph nodes when the tissues were subsequently treated with formalin, acidified with hydrochloric acid. Serial sections of the nasal region showed granules in the peri-neural spaces of the olfactory nerves, extending into the nasal mucosa and staining it blue. Here the granules lay beneath the epithelial cells in the meshes of loose connective tissue. In this tissue were thin-walled granule-containing vessels which Weed believed to be lymphatics. He concluded that a tissue space always lies between the peri-neural sheath containing cerebro-spinal fluid and the fine terminal vessels of the lymphatic system.

Certain aspects of Weed's work deserve special attention.

(a) He was careful to employ pressures 'only slightly in excess of physiological' (Details not given)

(b) The site of introduction of the solution was always in the lower thoracic or upper lumbar region. This focused attention exclusively on the cranial section of the sub-arachnoid space and made it impossible to investigate any outflow into lymphatics from the lumbar part of the sub-arachnoid space. It is just this region which Ivanow (1927) believed to possess a particularly rich lymphatic connexion.

(c) The Weed double salt mixture has been criticized by Spirov (quoted by Ivanow, 1927), who repeated Weed's technique on living dogs and dead human embryos. He observed that the salts impregnated the cranial bones, the bones and ligaments of the vertebral column and the cellular tissues around the blood vessels and nerves of the neck. This led Spirov to conclude that this crystalloid mixture could diffuse very rapidly through tissues and was no true indicator of preformed anatomical pathways.

(d) Weed carried out a large series of injections of suspensions of carbon granules into the spinal sub-arachnoid space, but always 'no granules could

be found on microscopic study in the cervical lymph glands and their channels' (1914, p. 80). This conflicts with the results of many subsequent workers and the disparity is probably due to the differences in the size of the particles used.

Woollard (1924) found trypan blue in the cervical lymph nodes of cats following its introduction into the cisterna magna, but dismissed this finding as being secondary to leakage from the puncture site.

Speransky and his co-workers began their investigation of the sub-arachnoid outflow in 1927, when Spirov repeated the work of Weed as already described. As a result of Spirov's findings, Ivanow (1927) employed India ink as the indicator substance. The introduction of the ink into the sub-arachnoid space via the lumbar route was commenced in the living dog and continued after its death for from 1 to 3 days. This led to a filling of the deep cervical, posterior abdominal and posterior thoracic lymph nodes, although the actual outflow channels were not evident. An exactly similar picture was obtained in the living animal after the introduction of 5 c.c. of ink suspension by the lumbar route.

A more detailed account of these channels was given by Ivanow & Romodanowsky (1928), who worked on the corpses of dogs and on living animals, the India ink being introduced by cisternal puncture or by laminectomy in the lumbo-sacral region. These workers concluded that

(a) After cisternal injection, India ink was seldom seen to move farther caudally than the mid-thoracic region.

(b) Peri-neural spread in the spinal nerves only occurred as far as the intervertebral ganglia. This was true for the fifth and seventh cranial nerves also. Only in the olfactory, optic and acoustic nerves did ink spread throughout the whole extent of the nerve.

(c) Ink was seen to pass into 'segmental' lymph vessels which arose from the surface of the dura at points where digitations of the ligamenta denticulata were attached.

(d) These lymph vessels filled only sharply demarcated portions of the deep lymph nodes.

Moreover, in all the above experiments it was the nodes at the bifurcation of the aorta which showed maximal filling, commencing soon after the cervical filling. After small injections and brief periods of survival, it was these two groups, the cervical and aortic, which alone contained ink.

Gilkin (1930), again using India ink, extended Ivanow's results by investigating the relative outflows from various isolated segments of the sub-arachnoid space under a perfusion pressure of 30 cm. of water. Cervical and lumbo-sacral outflows were found to be considerably in excess of that obtaining in the thoracic region.

Confirmation of a connexion between the sub-arachnoid space and the lymphatic system at all spinal levels was obtained by Oshkaderow (1936), who used India ink, Gerota mixture and thorium dioxide as injection masses. He also demonstrated lymph vessels passing from the arachnoid through the

atlanto-occipital membrane to lymph nodes below and behind the ear, and thence to the deep cervical lymph nodes

Since 1936 work has been concerned exclusively with the outflow from the cranial sub-arachnoid space and its relation to the nasal mucosa and the cervical lymph nodes. Such literature falls outside the scope of the present review as far as regional distribution is concerned, although it may possibly throw light on the mechanism of the outflow presently to be considered

Considerations of technique

(1) The nature of the indicator substance

This must be non-irritant and non-toxic. Estimation of the particle size is essential and in the India ink employed in the present work the particle sizes range from 0.4 to 1.5μ . The majority (more than 90%) are under 0.5μ . The form of the smaller particles is roughly spherical, while the larger ones are aggregates of more than one particle.

(2) Site of introduction of the indicator substance

The indicator substance must have access to the whole of the sub-arachnoid space. Moreover, there should be as little leakage of ink as possible from the introduction site. With these two provisos in view, the lumbar introduction as practised by Weed and the sub-occipital method of Woollard (1924) were rejected. Instead ink was introduced directly into the ventricular system or into the cranial sub-arachnoid space through a small burr hole which could be effectively plugged with bone wax.

(3) Control of injection pressure

The ink suspension was allowed to run in at a pressure not exceeding 120 mm of ink, and was only introduced after withdrawing an equal or rather greater volume of cerebrospinal fluid.

(4) Quantity of ink injected

By making repeated injections, as much as 4.5 c.c. of ink could be introduced into one animal.

MATERIAL AND METHODS

Animals

Adult rabbits of weights from 1.5 to 3.0 kg were used.

Anaesthesia

This would appear to influence materially the result of the experiment. Thus, in the earlier experiments of this series when urethane was employed and the animals did not recover consciousness, it was found that very little ink left the sub-arachnoid space. Later, with the use of sodium nembutal (2.5% intravenously, something less than 2.0 c.c./kg body weight) allowing of the recovery of the animal in about 2-2½ hr, the outflow of ink was markedly

increased. It would seem important then, that the animal should return to full activity as soon as possible after the operation, presumably in order that a normal level of cerebrospinal fluid pressure may be restored.

Preparation of the India ink

It is important to use good-quality India or Chinese ink in stick form. The stick of ink was rubbed down in double strength Ringer solution until a concentrated suspension was produced, then filtered through a no. 5 Whatman paper and sterilized immediately before use.

Technique

Approach to the lateral ventricle in the first five animals of the series was made by removing a temporal bone flap. The underlying ventricle was then approached through the area of brain thus exposed. This technique was abandoned because it was felt that the presence of a decompression caused by the removal of a bone flap might prevent the return of cerebrospinal pressure to normal and also because complete control of leakage of ink was found to be unattainable.

In later experiments a vertical incision was made half-way between the posterior border of the supraorbital ridge and the nuchal ridge of the occiput, and the temporalis muscle divided in the line of the incision after separating it from the bone with an eye spud. The bone was drilled with a dental drill of about 1 mm diameter, directed backwards at 20° to the coronal plane and downwards at 20–25° to the horizontal plane. To puncture the dura, to fuse effectively dura and arachnoid and to prevent bleeding from the dural vessels, a blunted needle at dull red heat was passed down the drill hole and just through the dura. A fresh sterile drill was placed in the hole to serve as a guide for the needle. The latter—a children's type lumbar puncture needle—was then swung into place over the hole and slowly introduced until clear cerebrospinal fluid could be obtained on slight suction. A 20 c.c. syringe containing that amount of ink was then attached to the needle and supported in a clamp. The ink ran in briskly at first but slowed up as the volume entering approached that of the cerebrospinal fluid withdrawn. The introduction usually took about 5 min. This method was particularly suitable for repeated injections as the same hole could be entered two or three times before changing to the other side. As the needle was withdrawn the burr hole was filled with bone wax. The animals were usually killed by exsanguination through the thoracic aorta under paraldehyde anaesthesia.

Subsequent examination showed that in many animals the needle had passed through the whole thickness of the occipital lobe and had entered the subarachnoid space on the dorsum of the brain stem. In these cases the amount of cerebrospinal fluid withdrawn was in excess of that which was normally obtained from true ventricular punctures. In so far as the pressure of introduction of the ink did not exceed 120 mm. of ink, and that the possibility of

leakage had been eliminated, such introductions were perfectly valid for the purposes of the present investigation

OBSERVATIONS

On laminectomy the most striking feature is the accumulation of India ink around the nerve roots of the lumbo-sacral and cervical regions. These accumulations are the 'ink-cuffs' ('*Tuschenmanchetten*' of Ivanow). They are less marked in the thoracic region.

On the dorsal nerve root the typical cuff takes the form of a truncated cone with base directed medially—up against the cord—and apex falling just short of the dorsal root ganglion. Whilst the position of the apex is constant in all

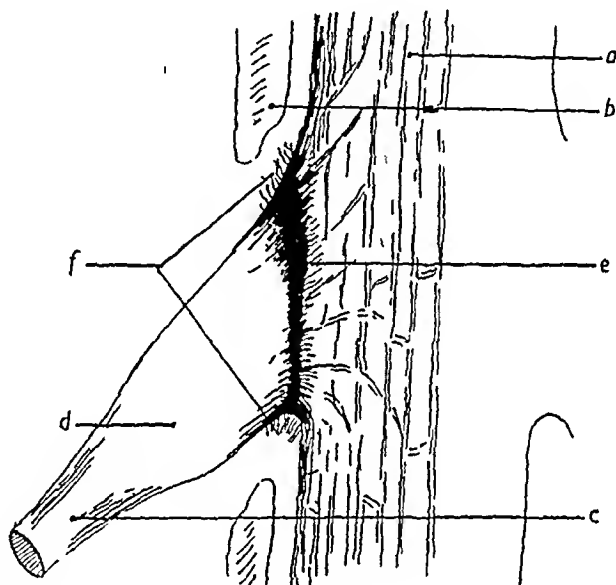


Fig 1 Ink cuff of upper sacral nerve a, dura mater, b, pedicle of vertebra, c, spinal nerve, d, dorsal root ganglion, e, ink cuff, f, fine black lines arising from ink cuff

cases, the exact position of the base is dependent upon the amount of accumulated ink—which in turn depends upon the amount introduced. From the base, thin black lines pass on to the cord apparently due to the presence of ink between diverging fibre bundles. In this way a fan-like effect is produced which may bear a superficial resemblance to a lymphatic network (Fig 1). Along the anterior nerve roots the accumulation of ink is less intense. Its distal limit is less defined, but is as a rule some 2–3 mm farther out along the nerve than is the rule in the dorsal nerve root.

Observation under the low-power binocular dissecting microscope showed a leash of fine black lines (? lymph vessels) passing from the region of the nerve root culs-de-sac ventrally towards the paravertebral lymph nodes. This appearance was again only well developed in the lumbo-sacral region.

In those animals that had received larger quantities of ink (2–4 c.c.) it was possible to see a few slender black lines passing from the ventral surface of the

dural tube to either side of the posterior longitudinal ligament at the point where it spans the large foramen in the posterior surface of the vertebral body. On the posterior abdominal wall, definite fine lymphatic vessels could be traced backwards from the ink-filled nodes to the mid-point of the vertebral bodies between the two psoas muscle masses. It seems evident that these vessels collect ink both from the ink-cuff and the ventral surface of the dural tube.

In certain animals it was noticed that the epidural fat was stained grey, particularly in the sacral region. Histological examination of the fresh material revealed India ink particles lying, not in vessels but free in the meshes of the fat.

The presence of ink in one or more of the cervical, posterior thoracic, posterior abdominal and pelvic lymph nodes was a constant finding in all animals of the series. The accompanying table indicates the distribution and intensity of the ink in the various lymph nodes, the animals being arranged in order of their survival times. With adequate filling it can be seen that ink accumulates most markedly in the nodes of the neck, those around the bifurcation of the aorta and in the nodes of the hollow of the sacrum.

The deep cervical nodes are the first to fill and are followed by a pair of nodes in front of the upper part of the sacrum. Ink is visible in the superficial cervical lymph nodes at approximately the same time as in those of the aortic bifurcation. The typical picture of the widely separated ink-containing nodes of the cervical and aortic regions is now complete. After an interval of 30-36 hr certain small nodes of the posterior body wall begin to exhibit ink. The first of these lies in front of the bodies of the lumbar vertebrae in the groove between the two psoas muscles. These nodes are always incompletely filled, such filling commencing in the posterior part of the node and being due to the arrival of ink particles along fine vessels which can be traced backwards to the mid-point of the body of each lumbar vertebra. Such vessels are strictly segmental, whereas the small nodes just described do not always qualify for this description as their number may not correspond to that of the related vertebrae. Nevertheless, 'segmental node' has been used as a term of convenience in this work.

In the thoracic region the first nodes to contain ink are those grouped together in the posterior mediastinum. The last nodes of the body wall to exhibit ink are two or three lying on the bodies of the thoracic vertebrae between the bifurcation of the trachea and the crura of the diaphragm.

Beyond the segmental vessels already mentioned, it was not possible to identify any other vessels that might be conveying ink particles from the sub arachnoid space to the lymph nodes. As it was, segmental vessels never showed more than a moderate greyness even in those animals that received the largest quantities of ink.

Histological examination of a lymph node in an early stage of filling showed much of the ink lying in the peripheral lymph sinus. At first it appears to be largely free, but later the number of macrophages containing ink granules increases.

Table 1 *Analysis of experimental results*

Animal	Weight (kg)	Anaesthetic	Approach	Vol of Ink (cc)	Injec- tions	Survival (hr)	Lymph nodes										
							Cervical		Thoracic		Lumbo sacral						
							Super- ficial	Deep	Medi- astinal	'Seg- mental', ventral'	'Seg- mental', dorsal'	Aortic	Sacral				
1124	2.6	Paraldehyde	Bone flap	0.8	1	1	O	+	O	O	O	O	+	+	+	+	+
1118	2.5	Paraldehyde	Bone flap	0.5	1	12	+	+	O	O	O	O	+	+	+	+	+
1123	1.5	Paraldehyde	Bone flap	0.5	1	17	+	+	O	O	O	O	+	+	+	+	+
1113	2.5	Paraldehyde	Bone flap	0.8	1	18	+	+	O	O	O	O	+	+	+	+	+
1114	2.4	Paraldehyde	Bone flap	0.5	1	22	+	+	O	O	O	O	+	+	+	+	+
1126	3.0	Paraldehyde	Drill hole	0.5	1	24	+	+	O	O	O	O	+	+	+	+	+
1127	3.5	Paraldehyde	Drill hole	0.8	1	25	+	+	O	O	O	O	+	+	+	+	+
1131	2.1	Nembutal	Drill hole	1.0	1	29	+	+	O	O	O	O	+	+	+	+	+
1130	2.1	Nembutal	Drill hole	1.0	1	36	+	+	O	O	O	O	+	+	+	+	+
1134	3.5	Nembutal	Drill hole	2.4	3	56	+	+	+	+	+	+	+	+	+	+	+
1128	2.4	Ether	Drill hole	1.0	1	57	+	+	+	+	+	+	+	+	+	+	+
1132	2.1	Nembutal	Drill hole	2.3	3	84	+	+	+	+	+	+	+	+	+	+	+
1129	3.0	Ether	Drill hole	1.0	1	98	+	+	+	+	+	+	+	+	+	+	+
1133	2.6	Nembutal	Drill hole	2.6	4	101	+	+	O	+	+	+	+	+	+	+	+
1136	2.4	Nembutal	Drill hole	4.4	5	108	+	+	+	+	+	+	+	+	+	+	+

Animals arranged in order of survival times

O = no ink in lymph node

+ = ink just recognizable macroscopically

+ + = enough ink present to give node dark grey colour

+ + + = enough ink present to give node black or almost black colour

DISCUSSION

The choice of India ink as the indicator substance in this work was made after careful consideration of the arguments levelled against it. Thus Weed believed that the channels used by the body for the removal of particulate material from the sub-arachnoid space might differ from those normally used for the absorption of the cerebrospinal fluid itself in so far as the former might involve a process of phagocytosis. Weed quoted his own experimental results in support of this argument, showing that sub-arachnoid introduction of a carbon suspension (particle size not stated) never resulted in the appearance of carbon in the cervical lymph nodes unless excessive pressure (100 mm Hg) was used. On the other hand, similar introduction of the ferrocyamide solution always allowed Prussian blue granules to be demonstrated in that site. The present investigation has failed to confirm this distinction, and with India ink of particle size $0.4-1.5\mu$, a marked and rapid filling of the cervical lymphatic system was constantly obtained. It follows then that the rigid differentiation maintained by Weed cannot be upheld and that both particulate and solute indicators may reach the lymphatic system.

In view of the apparent unimportance of phagocytosis as a means of transport, it must be concluded that the particulate suspension will collect at points of outflow of cerebrospinal fluid from the sub-arachnoid space. Thus marked aggregations of ink are always found around the olfactory bulbs and it is believed that the 'ink-cuffs' of the spinal nerves have a similar significance. Nevertheless, it must be recognized that the various solutes of the cerebrospinal fluid stand closer in the matter of diffusibility to 'crystalloids' than to particulate matter. Thus the routes taken by Weed's double salt mixture may in some respects indicate more accurately the physiological potentialities of solute migration.

The normal pigment of rabbit lymph nodes may cause some confusion with India ink, particularly to the naked eye. Microscopically, however, the distinction between pigment and ink is easily made as the former appears as golden yellow or yellow brown (intra-cellular) granules, whilst ink particles are jet black and are both intra- and extra-cellular.

The employment of injection pressure not exceeding 120 mm of ink cannot be in any way regarded as unphysiological, particularly as the ink merely replaces a corresponding volume of cerebrospinal fluid. It is to be noted, however, that the pressures used by Ivanow (1927), though well below those used by preceding workers other than Weed, ranged from 30 to 50 cm of water and it might be argued that, as such, they were excessive and likely to produce a false picture through damage to tissues. Nevertheless, his results are substantially similar to those obtained in the present series of much more 'physiological' experiments.

The more striking accumulation of ink in the 'ink-cuffs' of the cervical and lumbo sacral nerve roots is probably related to their relatively larger size. The

precise anatomy of the region is still disputed. A detailed but somewhat schematic description of the nerve roots between their emergence from the spinal cord and the level of the posterior root ganglion is given by Sicard & Cestan (1904), who termed this zone the '*nerf de conjugaison*'. They investigated its structure by means of Chinese ink injected into the sub-arachnoid space through the atlanto-occipital membrane and found culs-de-sac of both sub-dural and sub-arachnoid spaces. This latter they found to have no connexion with the sub-epineural space of the peripheral nerve. Ballooning of the sub-arachnoid cul-de-sac was found to be the initial result of increased cerebrospinal fluid pressure.

Two distinct mechanisms may be invoked to explain the high concentration of ink in the cul-de-sac.

(i) A partial 'filtration' of the cerebrospinal fluid at these points, the majority of the particles being retained as by a strainer or filter, whilst fluid itself passes on into the lymphatic system carrying with it passively a certain amount of ink which eventually accumulates in the regional lymph nodes.

(ii) Phagocytosis of ink particles by macrophages normally stationed in the neighbourhood of the culs-de-sac and subsequent migration of these cells to the lymph nodes. Little or no fluid transudation may be involved in such a process.

The observations already recorded show that the description of the pathway taken by ink particles from the outer surface of the dura to the 'segmental' lymph nodes is still incomplete. The important and most elusive link in the anatomical chain lies between the sub-arachnoid space and the exterior of the dural tube in the region of the 'ink-cuff'. To explain the passage of India ink through this 'dura-arachnoid' membrane, either in the region of the 'ink-cuff' or elsewhere, one or more of the following mechanisms may be invoked.

(i) The dura-arachnoid might possess stomata permitting the passage of particles into the epidural connective tissue whence they pass into fine lymph vessels. Such stomata have often been postulated but never satisfactorily demonstrated.

(ii) The dura-arachnoid might exhibit a special permeability in respect of particles in the region of the 'ink-cuff'.

Either of these two mechanisms would explain the presence of free ink in the epidural fat of the lower sacral region.

(iii) Particulate matter might be taken up by phagocytic cells of the dura-arachnoid and actively transferred to lymphatics on the outside of the dura. Such cells would therefore be present at the sites of maximal ink concentration, notably in the 'ink-cuffs'.

The relative unimportance of phagocytosis in this region is shown by the histological examination of the 'ink-cuff', where only a minority of particles are seen to lie within macrophages. Thus it would seem unlikely that phagocytosis is responsible either for the concentration of ink in the 'ink-cuff' as suggested above, or for its transport to the epidural tissue and beyond. It is to be noted, moreover, that microscopic examination of the lymph nodes

indicates that in the early stages of filling most particles lie free in the peripheral lymph sinus and only later become ingested by macrophages

Quincke (1872, pp 161-162), using cinnabar, also concluded that transport could not be due to carriage by macrophages, as in many experiments masses of fine cinnabar particles could be found at a considerable distance from the point of introduction

More recently much the same problem has arisen in connexion with the transport of foreign particles from the alveoli of the lungs to the regional lymph nodes The balance of evidence suggests that movement of such particulate matter is not brought about—at any rate exclusively—as a result of preliminary phagocytosis (Drinker & Yoffey, 1941, p 87) The rapidity with which transference takes place militates against the latter view, and the same point may be urged with regard to our findings as to the rapidity of appearance of ink in the cervical nodes after introduction into the lateral ventricle It is to be noted also that the size of particles employed in the present work was well below the limit of 2μ given by Gillilan & Conklin (1938) as capable of direct passage into lymphatic channels

It appears probable then, that the cuff is an area in which both fluid and particulate matter may leave the sub-arachnoid space through the thickness of the dura-arachnoid and so become epidural The local epidural fat or connective tissue becomes ink-stained, and it is in this epidural layer that blindly ending lymphatics arise and conduct fluid and particles to the regional lymph nodes This postulated pathway is summarized in Fig 2

The mechanism here outlined is comparable in its main features with Weed's (1914) reconstruction of the passage of the Prussian blue solution from the sub arachnoid space into the nasal mucosa and cervical lymph system (Fig 3) The tissue space which, this author maintains, always intervenes between the sub epineural space (sub-arachnoid prolongation) and the small blind lymph vessels, is represented in the above spinal scheme by the epidural space and its contained fatty connective tissue

Weed (1914) from the results of the ferrocyanide method observes that 'there is an obvious perineural deposit of precipitated granules which can be followed a short distance outward along the anterior and posterior nerve roots' (p 90), and again, 'From the perineural space about the spinal nerve roots absorption takes place along lymphatic channels' He concludes that this is the sole pathway for fluid escape from the spinal meninges (pp 90-91) Unfortunately, he presents no adequate anatomical basis for this important statement

The volume of fluid which may leave the sub-arachnoid space by lymphatic channels probably depends upon several factors Of these, the pressure of the cerebrospinal fluid is probably predominant, but this itself can be influenced in a number of ways—e.g. by the type of anaesthetic, the depth and duration of anaesthesia, variations in respiration and blood pressure, and possibly the functional conditions of the absorbing channels

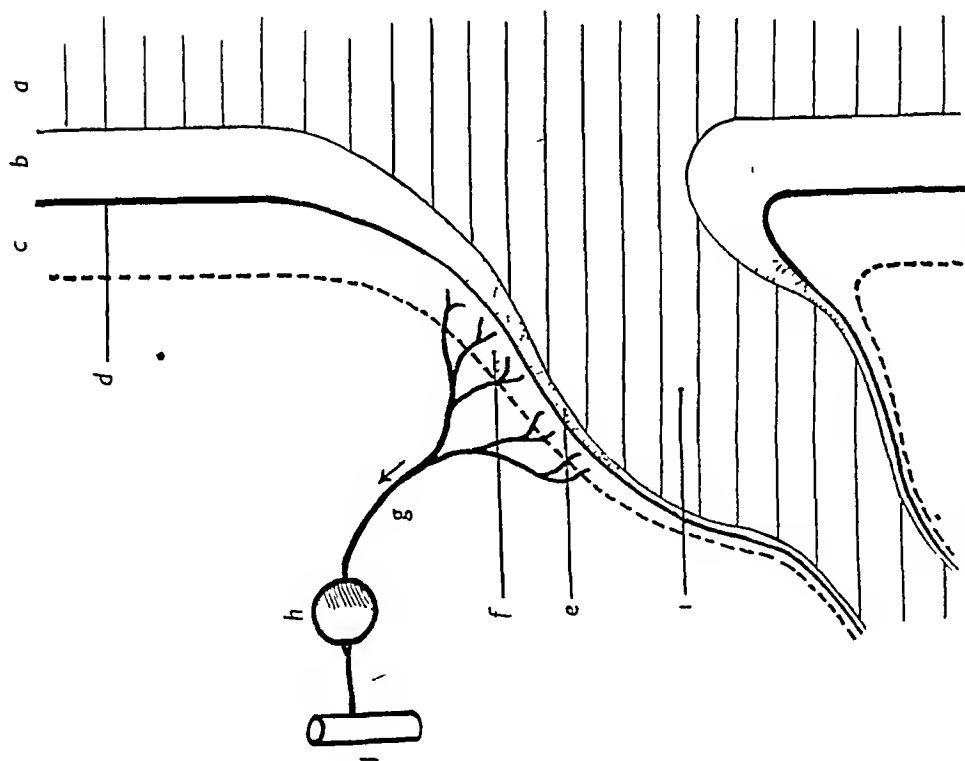


Fig 2 Diagram of spinal nerve root (rabbit) and its surrounding 'ink cuff'

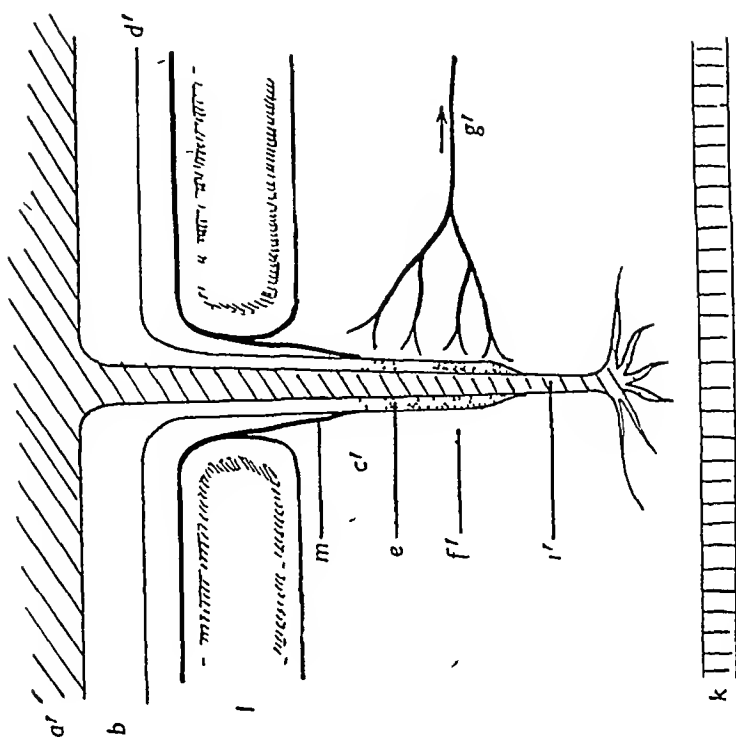


Fig 3 Diagram of olfactory nerve and its ink containing arachnoid sheath (rabbit)

The present communication has thus demonstrated that under conditions as nearly physiological as possible, particles not over 1.5μ can pass readily from the sub-arachnoid space not only into the cervical lymph nodes but into those lying in front of the vertebral column. These latter indeed may be regarded as the regional lymph nodes of the spinal sub-arachnoid space.

SUMMARY

1 The literature relating to the connexions between the spinal sub-arachnoid space and the lymphatic system is reviewed

2 A technique for introducing India ink into the cranial sub-arachnoid space of the rabbit without resultant rise in intracranial tension is described

3 Following such introduction ink appears within 4 hr in the nasal mucosa and cervical lymph nodes

4 Ink introduced into the cranial sub-arachnoid space can be found as low as the mid-thoracic level at the end of 1 hr and throughout the spinal sub-arachnoid space in 6 hr

5 Concentration of ink is marked around the lumbo-sacral nerve roots and in the terminal part of the spinal sub-arachnoid sac

6 Lymph nodes around the aortic bifurcation and in the hollow of the sacrum show ink filling in 6 hr

7 Repeated ink injection produces filling of the lymph nodes arranged along the front of the thoracic and lumbar vertebrae in a more or less 'segmental' manner

8 The pathway of this outflow is discussed

The authors are indebted to Prof J M Yoffey for his continued interest and encouragement, to Dr C F Powell of the Physics Department who carried out the estimation of size of India ink particles, to Dr H Heller of the Department of Pharmacology for repeated advice in experimental problems and to Mr Keith Hunt for the histological preparations

A supply of sodium nembutal anaesthetic was made available through the kindness of Abbot Laboratories

This investigation was made possible by the award to one of us (J B B) of a Research Scholarship by the British Medical Association of which grateful acknowledgement is hereby made

Lettering to Figs 2 and 3

a, spinal cord *a'*, olfactory bulb *b* sub arachnoid space *c*, epi dural connective tissue *c* nasal sub mucosa *d*, dura arachnoid (rabbit) *d*, arachnoid, *e* ink particles in sub arachnoid cul de sac *f*, ink particles in epi dural connective tissue *f*, ink particles in nasal sub mucosa *g* and *g*, fine lymph channels draining epi dural tissue and nasal sub mucosa *h* lymph nodes of body wall *i*, spinal nerve, *i'* olfactory nerve *j*, longitudinal collecting channel *l*, nasal mucous membrane, *l*, cribiform plate, *m*, dura mater

The observations described were made only on retinac, or portions of retinac in which injection was complete. An area showing no injected vessels was not considered to be avascular unless found so by the use of the high power of the microscope.

OBSERVATIONS

Adult Cat The major retinal vessels in the cat consist of three sets of arteries and accompanying veins, which run from the optic disc to the periphery, one pair nasally, one pair upwards and temporally, and one pair downwards and temporally. Pl 1, fig 1 illustrates the appearance of an injected retina mounted in glycerine. Between the temporal pair of vessels are several smaller ones running to the macula region. These vessels curve round the edge of the disc and are in communication for the most part with the choroidal circulation rather than with the central vessels of the optic nerve (Schultze, 1892). Otherwise, there is a great similarity between the retinal circulation of cat and man. The main vessels run in the nerve fibre layer, successive divisions of the vessels remaining at this level until the pre-capillaries are reached. When vein and artery pass each other the vein is always superficial to the artery—using the terms ‘superficial’ and ‘deep’ as synonymous with ‘inner’ and ‘outer’ as usually applied to the retinal layers. This has been observed in 170 crossings. The capillaries, as in man, fall into two groups, a superficial capillary net lying in the layer of nerve fibres, and a deep capillary net lying on the outer and on the inner aspects of the inner nuclear layer (Pl 1, fig 2). The precapillary vessels arising from the small arterioles, or arteriae afferentes, pass directly into the superficial capillary net and from the latter anastomotic vessels pass into the deep capillary net. Since many venous post-capillaries drain directly from the deep net, this may be considered to be more venous in character than the superficial net. It is unusual to find a pre-capillary passing into the deep net. In this respect the capillary circulation of the cat differs from that of man (Michaelson & Campbell, 1940) but resembles that of the rat (Hesse, 1880). The deep capillary net is in general more dense than the superficial. The average area of a capillary mesh of the deep net was found to be about 2000 sq μ and about 7000 sq μ in the superficial net with, however, great variations within both. Pl 1, figs 3 and 4 illustrate this difference in mesh size. Bruns (1882) found the capillary mesh diameter to vary from 40 to 90 μ . A striking feature of the capillary distribution is the absence, in a zone 100–200 μ wide on each side of the arterioles, of capillaries from the superficial net. A few capillaries of the deep net pass behind this zone, and these do not communicate with the arterioles (Pl 1, fig 5). The capillaries do not avoid the neighbourhood of the veins in a similar manner. In the macula region there is an almost completely avascular zone, having an average diameter of 300 μ . The peripheral margin of the retinal vascular system is formed by wide capillary arches joining the terminal branches of the arteries and veins. Between these arches and the ora serrata there is an avascular zone about 500 μ wide.

35-day embryo The optic disc and posterior portion of the hyaloid artery are illustrated in Pl 2, fig 6. It can be seen that no retinal vessels are present at this stage.

45-day embryo Several vessels can be seen proceeding from the edge of the disc into the retina for a distance of 0.12–0.24 mm (Pl 2, fig 7). With higher magnifications it can be seen that in the case of many vessels there is a solid column of cells extending for a short distance beyond the end of the Indian ink column. These probably represent the, as yet, uncanalized portion of the developing vessels.

51-day embryo The vessels have progressed farther towards the periphery (Pl 2, fig 8) and have now extended 0.36–0.72 mm from the optic disc. At places branches can be seen connecting the straight vessels and constituting early vessel loops, or vessel complexes. It must at this stage be only a conjecture that one limb of such a loop represents the future artery and the other the vein, as no difference in the walls of the limbs can be detected.

56-day embryo The vessel complexes which are three in number have developed in several respects (Pl 2, fig 9). They have grown farther towards the periphery, being 5.6, 4.80 and 4.56 mm respectively from the optic disc, and their more complicated arrangement is apparently the result of vessel growth from the limbs of the original loop described in the 51-day embryo. The limbs of the original loop can now be recognized as artery and vein in the light of the adult appearances and of the intermediate stages still to be described. At an average distance of 0.6 mm from the optic disc, where the main vessels begin to diverge from one another, a capillary system can be seen developing, although much of the retina remains still unvascularized. This capillary system shows several striking characters.

(a) By far the greater part of the developing capillary system is an outgrowth from the veins, and forms around them a triangular-shaped bed with base directed peripherally (Pl 3, figs 10, 11).

(b) The capillary growth from the vein is by a process of budding. These buds, which are placed at fairly regular intervals, have pointed growing tips (Pl 3, fig 12).

(c) If neighbouring artery and vein are close to each other the capillary growth is confined at first to the side of the vein remote from the artery. Pl 3, fig 13, at a higher magnification, $\times 40$, illustrates this feature quite clearly. As artery and vein diverge capillary growth takes place from the side of the vein towards the artery as well as from the far side. If a vein is situated midway between two arteries capillary growth occurs equally from each side of the vein.

(d) The embryonic capillary net formed at this stage differs from the definitive net in the greater size of the lumen and the smallness of the mesh. The net is entirely in the layer of nerve fibres. The large size of the lumen is illustrated in Pl 3, fig 14, though in this particular specimen the capillaries do not happen to be injected.

(e) The capillary net grows towards the neighbouring artery, but stops short of it so that a capillary-free area is left around the artery (Pl 3, fig 13) This zone is traversed by occasional single vessels which grow from the artery and, judging from their position, probably represent the future arteriae afferentes of the system These vessels are the products of the arteries and not of the surrounding capillaries, as evidenced by the occasional protrusion from the arteries of vascular buds which have not yet reached the neighbouring capillary bed (Pl 3, fig 13) At the periphery of the vascularized area the terminal venules and arterioles are linked up with each other by capillary arcades Around these arcades there is also a dense capillary development There is nowhere any evidence of capillary formation that is not a budding from pre-existing vessels

1-day-old kitten The development described in the 56-day embryo has now progressed to that illustrated in Pl 4, fig 15 The vessels have grown farther towards the periphery, being 5.04, 5.04 and 6.00 mm from the disc, and the non-vascularized areas around them are now much smaller than they were in the 56-day embryo The capillary bed round each vein retains the triangular shape noted in the 56-day embryo, and while the capillaries on the periphery of this triangular area retain their embryonic appearance, those nearer to the disc assume the character of the definitive reticulum with narrower lumen and broader mesh The capillary-free space round the arteries is more pronounced than in the 56-day embryo as the capillaries have grown more completely towards the arteries

8-day-old kitten The periphery of the vascularized area is now on the average 6.2 mm from the disc, and practically all of the retina is vascularized The capillaries near to the periphery are still of the primitive type

15-day-old kitten The periphery of the vascularized area is now on the average 6.7 mm from the optic disc In some places a deeper capillary network can be seen developing from the superficial net The peri-arterial capillary-free zone is fairly well demarcated (Pl 4, fig 16)

22-day-old kitten The vascularization of the retina which now extends about 7.20 mm from the optic disc is very similar to that of the adult stage except in two features (1) there are still capillaries of the primitive type in the peripheral areas, and (2) the deep capillary network has not yet formed completely The capillary-free zone around the arteries is well demarcated, almost better than in the adult cat (Pl 4, fig 17) This zone may even be found around the arteriae afferentes (Pl 4, fig 18)

The pattern of capillary development outlined above was found to be reproduced in all the embryos and in the different litters of kittens examined, with, however, some notable differences in the rate of development For example, of three 53-day embryos two showed development similar to that described above for a 51-day embryo, while one was similar to the 56-day embryo described, and a 28-day-old kitten belonging to a poorly nourished litter continued to show a large number of capillaries of the embryonic type (Pl 4, fig 19)

DISCUSSION

The present conception of development of the vascular system is that its primordium consists of a general capillary net preceding the formation of individual vessels (Krause, 1876, Evans, 1909, Sabin, 1920, Hughes, 1934, 1935, Hamilton, Boyd & Mossman, 1945) The endothelium of such a net is considered to arise from a syneytium of cells differentiated from the local mesenchyme (Finley, 1922, Sabin, 1920, 1922) The mesenchymal origin of vascular endothelium in the rabbit's choroid has been described by Fuchs (1905) Out of the capillary net so formed, the circulation develops certain broader and more defined pathways in accordance with haemo-dynamic forces first postulated by Thoma (1893) The course of development, according to these conceptions, may be summarized thus mesenchymal cell, angioblast, vascular endothelium, capillary, and finally artery and vein This method of vascular development is not present everywhere, since the heart itself is formed from chains of angioblasts rather than from a complicated plexus, and in the opinion of Federow (1910), supported later by Squier (1915), the pulmonary vein of the chick is produced at a very early stage of embryonic life as a proliferation of endothelium from the dorsal sinus wall which projects into the mesocardium Into this proliferation the sinus cavity tunnels, thus producing a short vessel which is the anlage of the vein The vein ultimately branches into capillaries which anastomose with capillary out-growths from the lung arteries Further, Buell (1922) found angioblasts coming directly from the wall of the sinus venosus of the chick and failed to find clumps of angioblasts unconnected with the mass, and therefore originating in mesenchyme

From the description given it appears evident that the vessels of the retina in the cat do not develop from a syneytium of cells differentiated locally but from vessels growing from the optic nerve head A syneytial origin would appear to be precluded by the absence of mesenchymal tissue in the developing retina The initial buds from the optic nerve head join to form loops, between the limbs of which a reticular capillary system appears The capillaries develop predominantly from the venous limbs of the loop, and to begin with from the side of the vein remote from the artery, if the artery and vein are close to each other These capillaries spread progressively in the nerve fibre layer from the vein towards the neighbouring artery for a well-defined distance, beyond which they do not go, leaving a capillary-free zone around the arteries in the nerve fibre layer This zone is traversed by arteriæ afferentes, while deep to the arteries a few vessels from the deep capillary net pass in the inner nuclear layer, and therefore about 45 μ from the artery The capillary-free zone lateral to the artery averages about 150 μ in breadth

The development of capillaries from veins, the early growth of the capillaries from the side of the vein remote from the neighbouring artery and the presence of a capillary-free space around the arteries are facts which appear to be

closely associated with each other and to be inherent in the process of capillary formation in the retina of the cat. These facts suggest that vascular development in the retina is not dependent on a morphological specificity inherent in the vessels. They rather indicate that this development is dependent on a factor or factors present in the retina and capable of effecting the budding of new vessels from the endothelium of veins. Moreover, they indicate certain attributes to the suggested factor.

The fact that the budding in the first instance occurs almost entirely from one side of the vein suggests that the growth factor is not in the venous blood plasma, as it is difficult to conceive of a factor within the plasma stimulating the endothelium on one side of the vessel only.

The fact that the capillary budding from the side of the vein nearer to the artery takes place later than that from the side remote from the artery suggests that the factor under consideration is present in a gradient of concentration such that it differs in arterial and venous neighbourhoods. This assumption seems to be supported also by the presence of a capillary-free space around the arteries.

The mode of spread from the veins laterally towards the arteries and the maintenance of a capillary-free space suggest that the factor determining the initiation of growth of the capillaries probably determines the distance to which it shall extend in a given time, initiation and cessation depending on variation in concentration of the suggested factor.

SUMMARY AND CONCLUSIONS

1 Vessel growth in the retina of the cat is by a process of budding from pre-existing vessels. No evidence is found of vascular differentiation from local cells.

2 The formation of retinal capillaries is pre-eminently a function of the retinal veins. Only the arteriac afferentes appear to originate from arteries.

3 If vein and artery are close to each other, growth takes place predominantly from the side of the vein remote from the neighbouring artery.

4 The spread of capillary growth towards an artery extends for only a certain distance, leaving a well-marked capillary-free space around the arteries similar to that present in other mammalian retinæ such as those of rat, dog, pig and man.

5 The anatomical facts are clearly associated with each other. Considered as a group they stimulate speculation regarding a factor situated in the non-vascular portions of the retina which may affect budding from the veins.

6 The present study of ontogeny shows that the bulk of the capillary system in the retina of the cat can be considered as part of the venous system. The arterial system is shown to be supra-capillary.

I have pleasure in acknowledging my indebtedness to Mr John Watt of this department for his expert technical assistance in the preparation and photography of the specimens.

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EXPLANATION OF PLATES

PLATE 1

- Fig 1 Appearance of injected retina of right eye of adult cat $\times 33$
- Fig 2 Section of injected retina of adult cat showing superficial capillary net in the nerve fibre layer and a deep capillary net lying on the inner and outer aspects of the inner nuclear layer $\times 130$
- Fig 3 Injected retina of adult cat showing the superficial capillary net $\times 100$
- Fig 4 Injected retina of adult cat showing the same area as illustrated in fig 3, but with the deep capillary net now in focus $\times 100$
- Fig 5 Injected retina of adult cat showing the area on either side of the artery free of capillaries from the superficial net. Deep to the artery, however, can be seen a few capillaries belonging to the deep capillary net. There is no capillary free zone around the vein $\times 35$

PLATE 2

- Fig 6 Injected retina of 35 day embryo. There are no vessels at the disc except the hyaloid artery $\times 3$
- Fig 7 Injected retina of 45 day embryo. Several vessels can be seen proceeding from the disc for a distance of 0.12-0.24 mm. $\times 43$

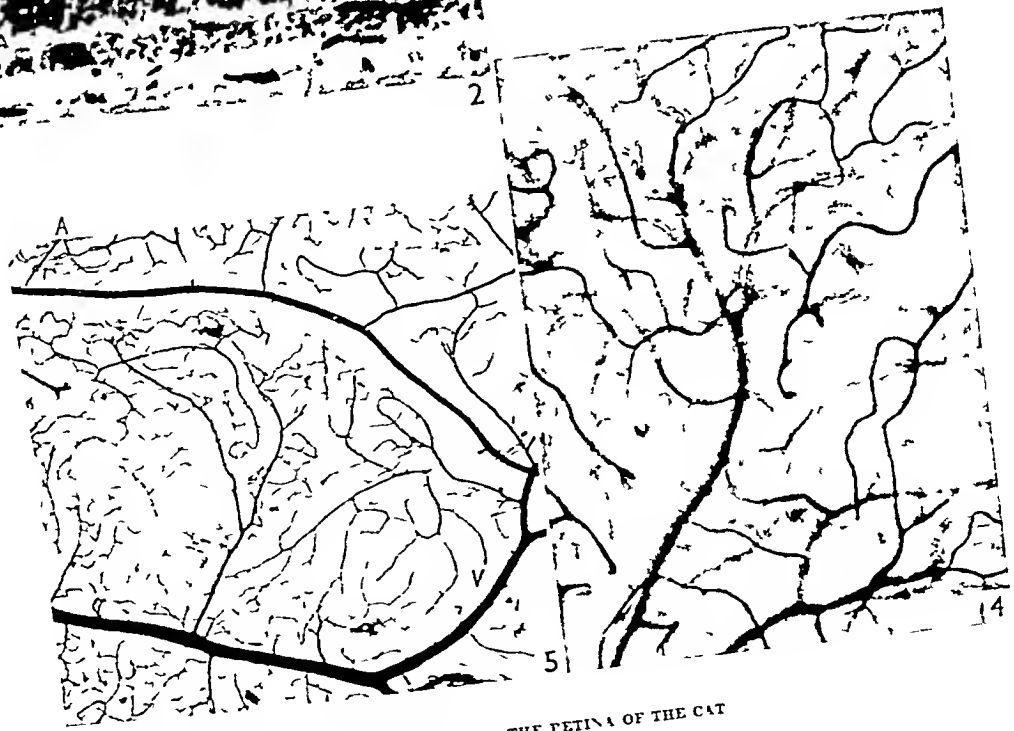
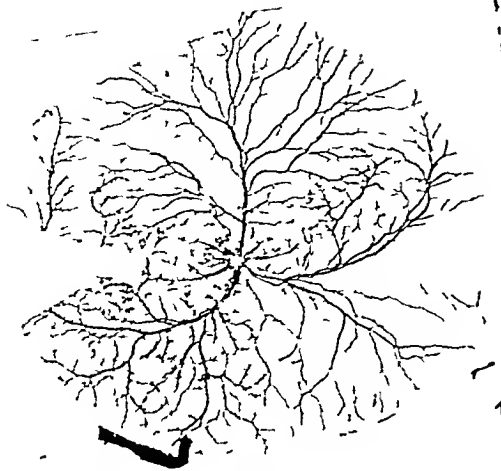
- Fig 8 Injected retina of 51 day embryo The vessels have proceeded from the disc for a distance of 0.36–0.72 mm The early formation of loops, although clearly seen with the microscope, are not reproduced in the photograph $\times 4.3$
- Fig 9 Injected retina of right eye of 56 day embryo Capillary growth is taking place predominantly from veins and in many places from the side of the vein distal to the neighbouring artery $\times 7$

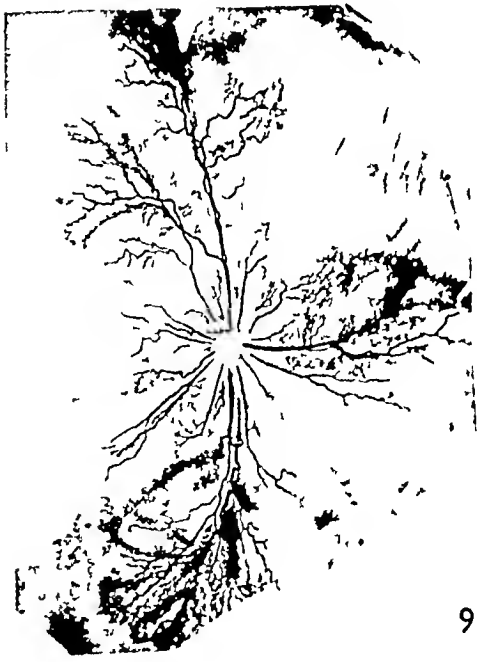
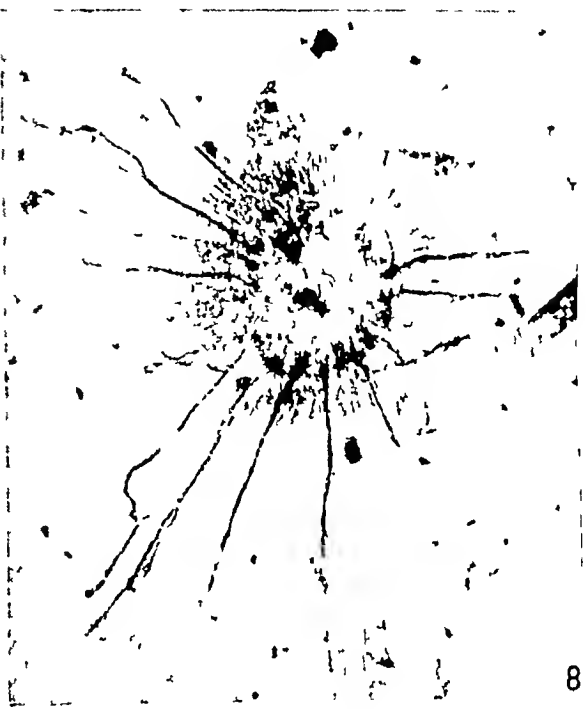
PLATE 3

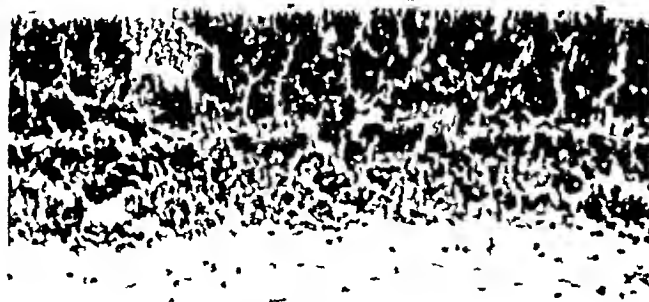
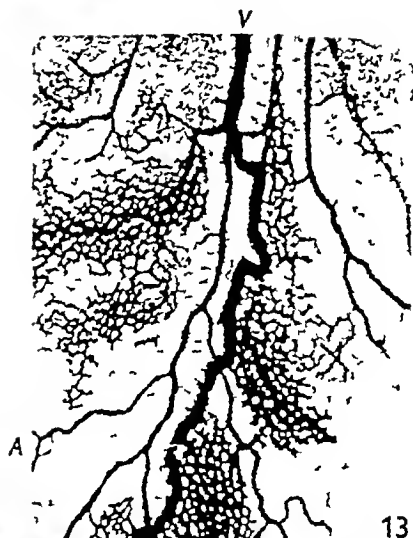
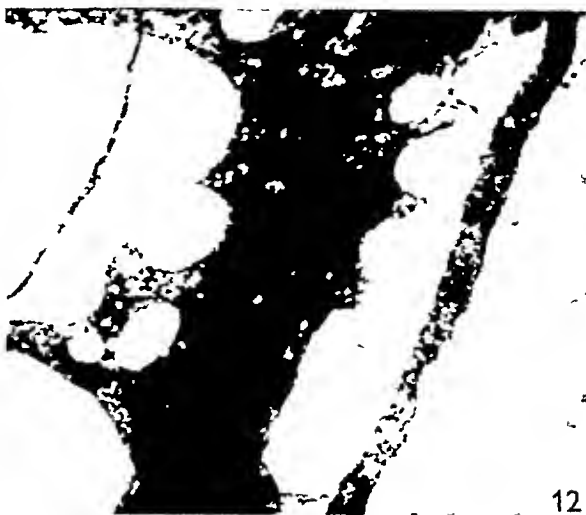
- Fig 10 The nasal vessel complex of the retina illustrated in fig 9 It shows that the growth of the capillaries takes place chiefly from veins and in places from the side of the vein distal to the neighbouring artery $\times 11$
- Fig 11 The lower temporal vessel complex of the retina illustrated in fig 9 It shows that the growth of capillaries takes place chiefly from veins and in places from the side of the vein distal to the neighbouring artery $\times 11$
- Fig 12 Vein and artery in injected retina of 56 day embryo The capillary growth is from the vein by a process of budding $\times 200$
- Fig 13 From injected retina of 56 day embryo (same as fig 11) showing capillary growth from veins and its confinement to the side of the vein away from the artery The capillary growth can be seen taking place fairly equally from each side of a small venous branch which is situated nearly midway between two arterial branches The early formation of a peri arterial capillary free zone can be seen, as well as the arterial precapillaries which traverse this zone These precapillary vessels are budding from the arteries $\times 40$
- Fig 14 Section of retina of 56 day embryo showing the capillary not placed entirely within the nerve fibre layer The capillary diameter is greater than in the fully developed eye This happens to be from a retina in which the capillaries were not injected $\times 200$

PLATE 4

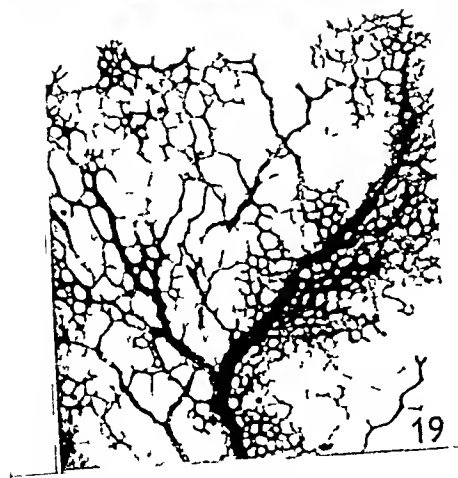
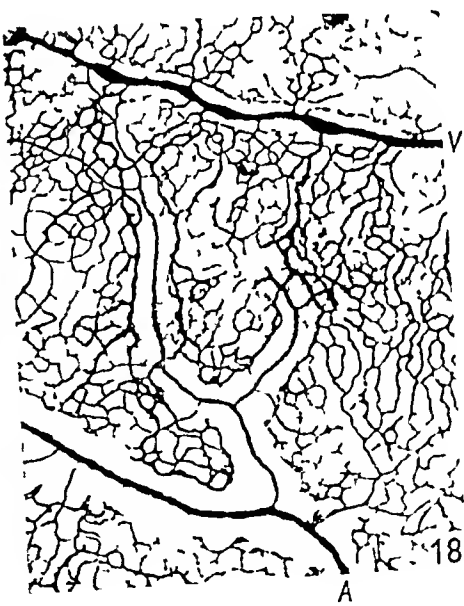
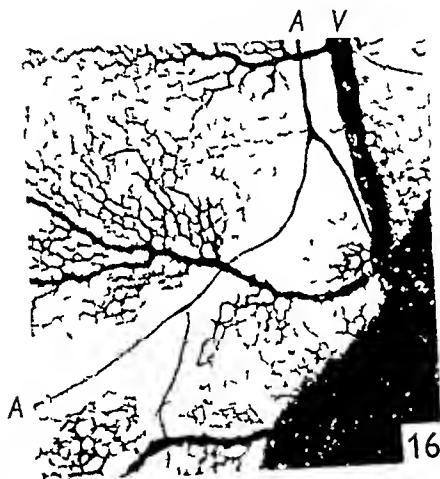
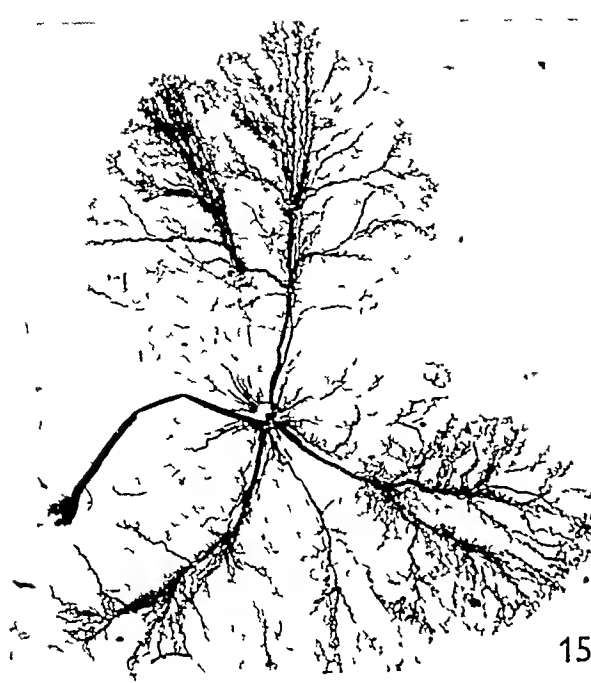
- Fig 15 Injected retina of 1 day old kitten $\times 7$
- Fig 16 Injected retina of 15 day old kitten showing the peri arterial capillary free zone The capillaries are concentrated around the veins and at places a deep capillary net can be seen forming $\times 30$
- Fig 17 Injected retina of 22 day old kitten showing a well formed peri arterial capillary free zone $\times 40$
- Fig 18 Injected retina of 22 day old kitten showing the peri arterial capillary free zone even around the arteriae afferentes $\times 40$
- Fig 19 Injected retina from a 28 day old kitten which was badly nourished Many of the capillaries are of the embryonic type as illustrated Note the capillary free zone around the arteries $\times 30$







Nerve fibre
layer



OBSERVATIONS ON THE INITIAL STAGES OF
OSSIFICATION *IN VITRO*

By H. RODOVÁ,* *From the Strangeways Research Laboratory,
University of Cambridge*

INTRODUCTION

The formation of bone in tissue cultures was first recorded by Fell (1928) and has since been described by several workers in different types of osteogenic tissue *in vitro* (Dolsechansky, 1929, Fell & Robison, 1929, 1930, Friedheim, 1930, Fell, 1932, Murray, 1934, Gaillard & Querido, 1939, Glücksmann, 1938, 1939, Miszurski, 1939).

The first stage in ossification is characterized by the appearance of a delicate network of osteogenic fibres among the osteoblasts. Another important feature is the production of alkaline phosphatase which, as Fell & Robison (1930) have shown by ordinary biochemical methods, occurs very early in the differentiation of osteoid tissue. The present study concerns this initial stage of ossification *in vitro* and has been made with special reference to the relationship of the cells to the intercellular fibres, and to the localization of alkaline phosphatase as demonstrated by Gomori's histochemical method.

MATERIAL AND METHODS

The tissue culture technique was based on one devised by Fell (1932) and modified by Glücksmann (1938). Explants were made of the frontal bone and, for control purposes, of the heart from 11–13 day chick embryos. The cultures were grown by the hanging drop method on $1\frac{1}{4}$ in square coverslips over $3 \times 1\frac{1}{2}$ in hollow ground slides. About 300 cultures were used for the investigation.

The procedure was as follows. A small square of bone was explanted in a mixture of 1 drop of cock's plasma and 1 drop of concentrated extract of the embryo from which the bone was obtained. After 2–3 days' incubation the culture was excised and transferred to fresh medium consisting of 2 drops of plasma and 1 drop of concentrated extract of an 11–13 day embryo. The tissue was allowed to grow for a further 2 days after which the original bone was cut out, leaving the broad zone of outgrowth untouched in the plasma extract clot. This ring of tissue was washed *in situ* with a drop of Tyrode's solution which was sucked off and replaced by a large drop of fresh medium, composed of 2 parts plasma 1 part embryo extract. A large drop was used to prevent a too great dilution of the medium by the traces of saline remaining on the culture, before it clotted, however, most of the mixture was pipetted off leaving only a thin layer in the central hole. The cultures were then

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re-sealed and returned to the incubator. Almost at once cells began to wander into the central space where growth was almost entirely on the surface of the coverslip, this rendered the living material very favourable for observation and photography. The cultures were studied for about 3 days after which they began to regress and were discarded.

The living tissue was examined with ordinary transmitted light, polarized light and Zernicke's phase-contrast illumination. The relationship between cells and fibres was investigated by taking serial photographs on cine film of cultures illuminated by Zernicke's method, the pictures were made at intervals of 4 min.

For histological study the cultures were washed in Tyrode's solution at 37° C to remove excess serous fluid and were then fixed either in Zenker's fluid without acetic acid, or, for the demonstration of alkaline phosphatase, in 80% alcohol. After dehydration and clearing in xylol, the cultures were examined as whole mounts, for the preparations were sufficiently thin for sections to be unnecessary.

It was not easy to find a suitable staining technique to demonstrate the osteogenic fibres in such whole mounts. After various methods had been tried, good results were obtained by first staining the tissue with Ehrlich's haematoxylin, then mordanting for 20–30 min in 1% phosphotungstic acid and finally counterstaining for 15 min in a modification of Mallory's aniline blue solution, in which the aniline blue was reduced to a quarter of the usual amount. In the finished preparations the cytoplasm was violet, the nucleus orange and the fibres blue.

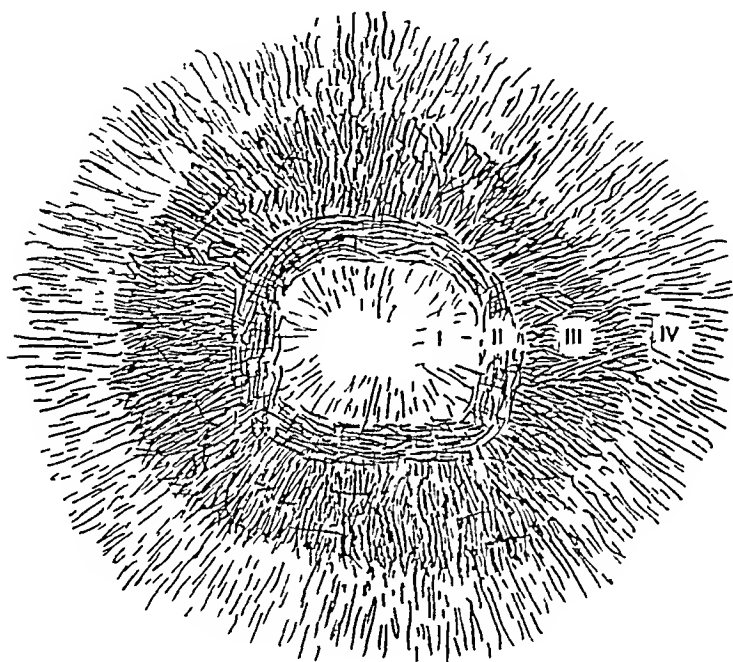
The histochemical method devised by Takamatsu (1939) and Gomori (1939) was used to demonstrate alkaline phosphatase. By this technique sites of phosphatase activity are rendered grey or black. After 2 hr fixation in 80% alcohol, the cultures were washed for about 15 min in distilled water. One group was then incubated in Gomori's sodium- β -glycerophosphate solution while another group, which served as a control, was incubated in the same solution without the glycerophosphate. Thirty minutes' incubation in the glycerophosphate solution was sufficient to give a positive reaction, but a better result was obtained after 2–4 hr. The subsequent treatment of the cultures was the same as that described by Gomori, the preparations were not counter-stained.

RESULTS

I *The development of the intercellular fibres*

For convenience of description the ring-shaped cultures may be subdivided into four zones (cf Text-fig 1) zone I, consisting of the ingrowing cells and fibres which eventually fill the central hole left by the removal of the original explant, zone II, a compact region immediately adjacent to zone I, in which the cells and fibres have a concentric orientation, zone III, a relatively broad, thick tract of tissue with no definite structural orientation, zone IV, the peripheral zone of outgrowth, composed of undifferentiated amoeboid cells.

Soon after the excision of the bone, cells begin to wander into the hole and by the end of the first day they usually occupy about one-third of the central space. A fine fibrous network rapidly appears among the cells many of which are larger, more expanded and less regular in outline than typical fibroblasts *in vitro*. The immigrating cells continue to spread and multiply and the intercellular fibres to increase in number and thickness, so that after 2 or 3 days' growth the central hole has become filled with a thin sheet of cells and fibres.



Text fig 1 Diagram of a ring shaped osteoblast culture showing the four zones

When zone I is viewed with polarized light the fibres, which are birefringent, are very distinct and are readily distinguishable from the cell processes, although the processes also are birefringent, their orientation is seldom the same as that of the neighbouring fibres and they can easily be traced back to the cell to which they belong. There is no evidence of any structural connection between the cells and fibres.

With phase contrast illumination the intercellular fibres which are beautifully clear, appear dark grey or black (Pl 1, fig 1 a, b) and serial photographs show that the cells move freely among them (cf Pl 1, fig 1 a, b). The fibres also alter their relative positions, probably in response to shifting tensions in the tissue, the fine ones become increasingly distinct and at the same time more numerous.

In favourable fixed preparations stained with haematoxylin and the aniline blue orange G solution, the fibres in zone I are clearly shown as a blue web over and between the cells with here and there longer and stouter fibres running in various directions. The fibres are sharply differentiated from the protoplasm of the cells. The cytoplasm of many of the osteoblasts contains granules which stain blue.

As stated above, the closely packed cells and fibres of zone II have a concentric arrangement which is probably due to the centrifugal retraction of the clot when the bone fragment is removed. The longest and stoutest fibres develop in this region (Pl 1, figs 2, 3), sometimes arising from the neighbourhood of a small fragment of the original bone (Pl 1, fig 3). They are very distinct both in living cultures viewed by ordinary transmitted light or with phase-contrast illumination, and in fixed preparations stained with haematoxylin and aniline blue. The tissue is too thick, however, for study with polarized light.

Only the stoutest fibres in zone III can be seen in the living cultures, owing to the thickness of the tissue in this region, but the histological structure is usually clear in the fixed tissue stained with haematoxylin and aniline-blue solution. In such preparations fibres of all sizes and stages of development can be seen running in every direction, some of the fibres are straight, others bent and their appearance often suggests that they may enlarge by the apposition of smaller fibrils. Fibres are rare in zone IV where the cells, being embedded in the old culture medium, are usually very fatty and somewhat degenerate.

The degree of differentiation that the cultures attain is closely correlated with the physiological state of the cells. Thus if cultural conditions are slightly unfavourable, as indicated by a slow ingrowth of cells into the central hole, distinct intercellular fibres are not formed. Under rather better conditions, a few fibres appear in the denser parts of the culture but not in zone I. Even if the tissue is growing vigorously, fibres do not develop if the cultures are embedded in too thick a clot so that the cells are rather fatty. The differentiation described above occurs only when the cells are in a very healthy active state.

II *The distribution of alkaline phosphatase*

The cultures give an intense reaction for alkaline phosphatase (Pl 2, fig 4) in preparations made by Gomori's histochemical method. This technique shows very clearly that two types of cell are present: cells resembling fibroblasts and differentiated osteoblasts. The cells of the fibroblast type are somewhat similar to those of ordinary embryonic heart cultures (cf Pl 2, figs 5, 7). The highest concentration of the enzyme is located in the nucleus, and the nucleoli and chromatin granules blacken after a very short incubation in the glycerophosphate solution. On the other hand, the cytoplasm gives only a very slight reaction. Whether the cells of the fibroblast type are true fibroblasts or 'dedifferentiated' osteoblasts is not certain.

The differentiated osteoblasts (Pl 2, fig 5) are very different in appearance from the fibroblast type of cell. They are much larger, of polygonal outline with many processes, and the whole cell gives an intense phosphatase reaction. Although the nucleus is the most strongly reacting part of the osteoblast, the cytoplasm also has a very high phosphatase content unlike that of fibroblasts.

During mitotic division the chromosomes in both types of cell evince great phosphatase activity and the cytoplasm also darkens, though a nearly colourless region is sometimes observed around the chromosomes (Pl 2, fig 6), there may be a blackening of the centrosomes. In both vegetative and mitotic cells the cell membrane is very distinct with Gomori's method.

The fine intercellular fibres also blacken with Gomori's method and may be seen running over and between the cells, the larger fibres, however, give no reaction. Clumps of osteoblasts without fibres are sometimes encountered, but not fibres in the absence of cells.

Zones II and III sometimes give a very intense reaction (Pl 2, fig 4), but the tissue is too dense for detailed study.

DISCUSSION

This study of the initial stages of bone-formation *in vitro* supports the view that the fibres are entirely extracellular and have no structural connexion with the osteoblasts. This is indicated by the following observations: (1) the cells can move freely among the newly formed fibres, (2) the fibres in the living tissue appear quite distinct from the cytoplasm when viewed with polarized light, (3) in suitably stained preparations the cells and fibres are sharply differentiated by their contrasting coloration. In some of Glücksmann's endosteal cultures a network of fibres projecting far beyond the zone of immigrating cells developed in the central hole, this was not seen in my preparations, probably owing to the fact that a different type of bone explant was used for the two investigations.

Although the osteogenic fibres appear to be entirely extracellular, the results of these experiments emphasize the fact that there is an intimate correlation between the physiological state of the osteoblasts and their ability to form fibres. As described above, if the cultures are growing weakly or if, though growing actively, they are too deeply embedded in the clot and are somewhat fatty, few if any fibres develop in the tissue. The effect of nutritional and other environmental factors on fibre-development will be investigated further.

Exactly how the osteogenic fibres are formed is not yet known. Direct observations on fibre-formation in living connective tissue *in vivo* have been made by Stearns (1940) using the rabbit ear chamber technique. Her results indicate that the intercellular fibres arise extracellularly as a result of fibroblastic activity but that the fibroblasts participate directly in the process by projecting from their surface cytoplasmic material which is apparently utilized in the production of fibrils.

It seems certain (Levi, 1931, 1932, Oliver, 1933) that connective tissue fibres which develop in culture are not associated with the fibrin of the plasma clot,

since they develop in serum also Stearns also finds no evidence of formation of fibres from a transformation of fibrin in the rabbit ear Levi suggests that the fibres are probably produced by gelation of the serum under the influence of some substance secreted by the cells

The question arises as to whether alkaline phosphatase is concerned in some way with the formation of the osteogenic fibres as well as with the subsequent calcification of the osteoid matrix As stated above, the enzyme is produced at a very early stage of osteogenesis (Fell & Robison, 1930) and Gomori's histochemical method has demonstrated considerable phosphatase activity in healing wounds during collagen regeneration (Fell & Danielli, 1943, Danielli, Fell & Kodicek, 1945) although the normal dermis gives almost no reaction Not only the fibroblasts but also the young fibres of the scar tissue are strongly positive, and it is interesting that in the osteoblast cultures also the fine fibres surrounding the differentiated cells react intensely with Gomori's method On the other hand, the fibres in the scar cease to react after they have reached a certain stage of development, as also do osteogenic fibres in the cultures

In general the presence of the enzyme seems to be associated with the differentiation and function of cells rather than with their multiplication (Bourne, 1943, Moog, 1944, Brachet, 1947) Thus in healing wounds in vitamin C-deficient guinea-pigs, alkaline phosphatase is nearly absent and no normal collagen fibres are formed, but the fibroblasts multiply actively (Danielli *et al.*, 1945)

The localization of the phosphatase in the cells is interesting Willmer (1942), using cultures of heart fibroblasts, was the first to describe its distribution in cells *in vitro*, and states that the reaction with Gomori's method is most intense in the nuclei and the chromosomes The high phosphatase content of chromosomes has been observed in other tissues (Krugels, 1942, Danielli & Catcheside, 1945) My own observations on the localization of the enzyme in fibroblasts *in vitro* fully agree with those of Willmer In the differentiated osteoblasts the reaction is much stronger, especially in the cytoplasm, than in the fibroblasts Whether the cytoplasmic phosphatase of the osteoblasts is identical with that in the nuclei and chromosomes is not known

SUMMARY

1 The initial stages of ossification *in vitro* have been studied in osteoblast cultures derived from the frontal bone of 11-13-day fowl embryos

2 Special attention was paid to the development of the osteogenic fibres and the distribution of alkaline phosphatase in the tissue

3 The relationship between the osteoblasts and the fibres was investigated in the living tissue by ordinary transmitted light, Zernicke's phase contrast illumination and polarized light, and in fixed and stained cultures mounted whole

4 The results supported the view that the fibres are entirely extracellular in origin

5 There was a close correlation between the physiological state of the cultures and their ability to form fibres

6 In preparations made by Gomori's histochemical method two types of cell, a fibroblast type and differentiated osteoblasts, are readily distinguishable

7 The nucleus of the fibroblast type of cell has a high phosphatase content but the cytoplasm is nearly negative. In the osteoblasts, which are much larger and more irregular in shape than the fibroblasts, both nucleus and cytoplasm react intensely

8 The possibility that the alkaline phosphatase may be concerned in the formation of the osteogenic fibres is discussed

I have pleasure in acknowledging my indebtedness to Dr H. B. Fell for much good counsel and guidance, to Dr A. Glücksmann, Dr M. Swann and Dr A. Hughes for assistance, advice and criticism, and to Mr V. C. Norfield for photomicrographs

I am especially grateful to the Czechoslovak Ministry of Education for the financial support which enabled me to study in England

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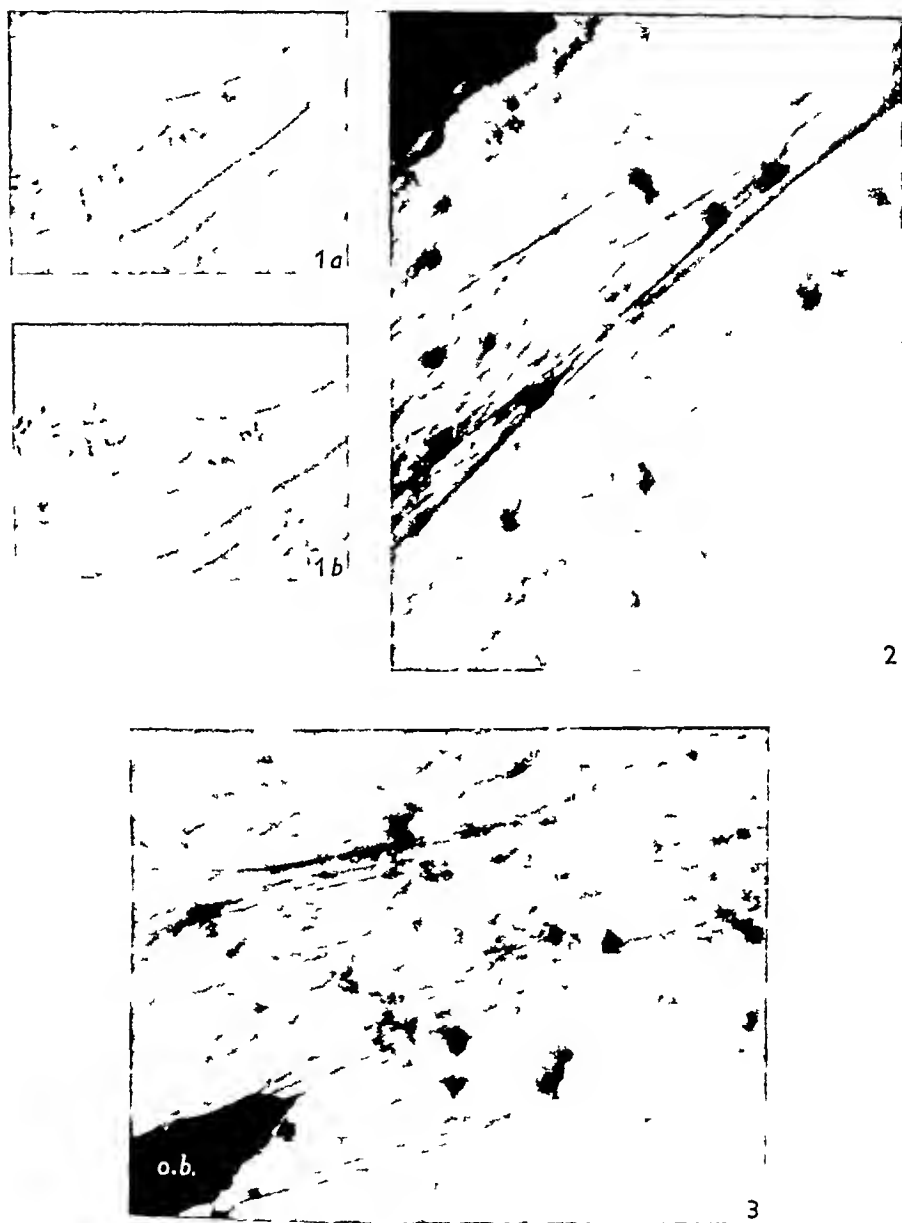
EXPLANATION OF PLATES

PLATE 1

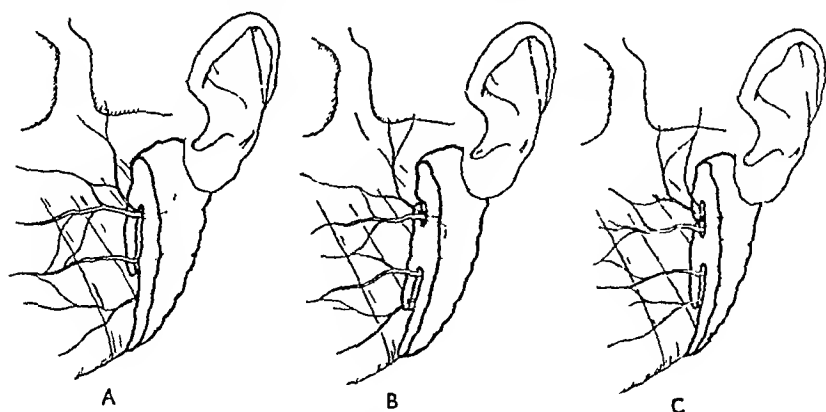
- Fig 1 (a) Culture of frontal bone from an 11 day fowl embryo, 2 days after removal of the original bone. A group of living cells and fibres have been photographed by Zernike phase contrast illumination ($\times 420$)
- (b) The same field after 58 min incubation. The relative positions of the cells and fibres have changed and one fibre has become thicker ($\times 420$) (Photographs by Dr A F W Hughes)
- Fig 2 Culture of frontal bone from a 12 day embryo, fixed 2 days after removal of the explant. Note the long, concentric fibres formed in zone II (cf Text fig 1)
- Haematoxylin, aniline blue orange G ($\times 330$)
- Fig 3 Same culture as that seen in Fig 2. In this region the new fibres are associated with a small fragment of the original bone (o b) ($\times 330$)

PLATE 2

- Fig 4 Culture of frontal bone from an 11 day embryo, treated with the Gomori Takamatsu method for the demonstration of alkaline phosphatase. Note the blackening of the tissue, indicating the presence of the enzyme ($\times 10$)
- Fig 5 Part of zone I in the culture illustrated in Fig 4, showing differentiated osteoblasts giving a strong reaction for alkaline phosphatase, and smaller, less reactive cells resembling fibroblasts ($\times 400$)
- Fig 6 Culture of frontal bone from an 11 day embryo, fixed after 24 hr incubation and treated for the demonstration of alkaline phosphatase. A differentiated osteoblast in metaphase is shown. The entire cell gives a strong reaction, which is particularly intense in the chromosomes ($\times 850$)
- Fig 7 Culture of the heart of an 11 day embryo, fixed 2 days after the removal of the original explant and treated for the demonstration of alkaline phosphatase. The fibroblasts are smaller and give a less intense reaction than the differentiated osteoblasts (cf Figs 5, 6), the nuclei of the fibroblasts react more strongly than the cytoplasm ($\times 400$)



in Gray and Cunningham, but that Bailey himself is inaccurate in his description of the gland as a bilobed structure, and in likening the relation of the facial nerve in the gland to that of the meat in a sandwich



Text fig 1 The configuration of the gland according to (A) Gregoire (1912), (B) McWhorter (1917), and (C) Rouvière & Cordier (1934)

METHOD

In the operation of parotidectomy the first step is to identify the branches of the facial nerve as they emerge from the borders of the gland, and then to dissect backwards along them in the plane between the deep and superficial parts of the gland. But small ducts, indistinguishable from fibrous tissue, may be present, linking the two parts of the gland and passing between the small branches of the facial nerve, i.e. at places other than between the two main divisions of the nerve. Unless these ducts are obvious, the surgeon cannot be sure that they are not being severed in this procedure of removal of the gland.

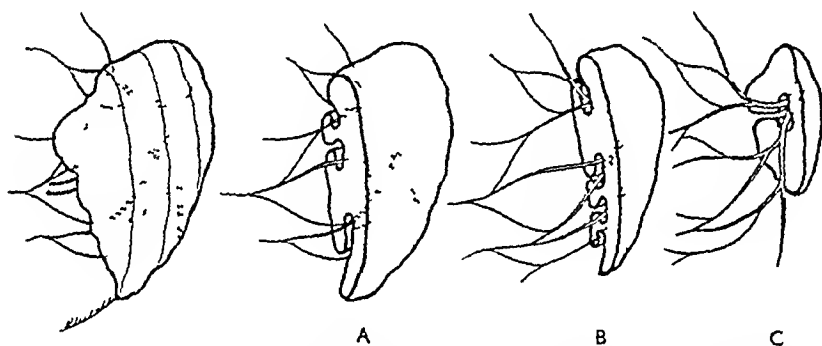
My technique, therefore, was to inject a coloured solution into the main parotid duct in order to ensure that all the connexions between the deeper and more superficial parts of the gland should be identified. At first I used Janus green incorporated in a cold gelatin mass, but later a diluted suspension of Indian ink was used, to avoid the possibility of the dye passing through the wall of the duct into the tissue spaces. The next step was, with the aid of a dissecting microscope, to identify and remove separately each lobule of the gland until the tributaries of the duct had been traced along their whole length. One full-time foetal gland and five adult glands were investigated in this way.

OBSERVATIONS

There is no doubt that deep and superficial parts of the gland can be identified, and that they are connected by glandular tissue which may be called an isthmus. There are, however, not one but many such isthmi. None of the specimens demonstrated the simple bilobed arrangement. Certainly, an obvious connexion always existed between the superficial and deep portions of the gland, and it did lie in the bifurcation of the main nerve. But not all of the tissue, lying deep

to the nerve plexus, drained towards the 'isthmus' situated between the two main divisions of the nerve, for in each of the adult specimens small ducts were observed passing between the small branches of the facial nerve. The majority of these ducts drained lobules of the gland lying deep to the nerve, and carried their secretion to a larger duct in the superficial part of the gland (Pl 1). They occurred in relation to both the temporo-facial and cervico-facial divisions of the nerve, although more numerous in the region of the latter (Text-fig 2A and B). A few of these ducts, however, ran in the opposite direction, i.e. they drained lobules lying superficial to the nerve and joined ducts in the deep portion of the gland.

In one of the adult glands, a group of lobules was seen surrounding a branch of the nerve just as a hand would grasp it (Pl 1). The foetal gland showed such a condition very well, and it was practically impossible to separate this part of the gland from the nerve without damaging the lobules. Although the interdigitating of gland and nerve could not be defined so readily in the foetus, this



Text fig 2 Three coronal sections of one specimen (from the present series of dissections) illustrating the complex relationship of nerve to gland

same specimen showed a fairly large duct running most of its course superficial to the nerve, but finally dipping between the two highest branches of the cervico-facial division to join the main duct in the deep part of the gland.

In all the dissections, at the upper and lower poles, parenchyma from the superficial portion of the gland burrowed into the depths, between the mandible and mastoid process, both above and below the main trunk of the nerve so as to encircle it almost completely (Text-fig 2C).

Each specimen had something different to show in the way of detailed distribution of the gland around the nerve, just as much as there was variation in the shape and extent of the whole gland and in the pattern of the plexus formed by the nerve.

CONCLUSIONS

The dissections show that communications between the superficial and deep portions of the gland may occur through any gap in the plexus of the facial nerve and, further, that the exact manner in which the gland interdigitates with

the nerve is not constant. We should, therefore, regard the parotid gland not as a simple bilobed structure but as a gland which, developing as an outgrowth from the buccal cavity, has travelled backwards in the direction of the ear and wrapped itself progressively around the branches of the facial nerve. A better analogy than a parotid sandwich would be to compare the gland to a creeper weaving itself into the meshes of a trellis-work fence.

Even the possibility of being able to define lobes of the gland in the living subject is very remote, although the surgeon may get this erroneous impression due to the difficulty of distinguishing the ducts as they traverse the plane of the nerve.

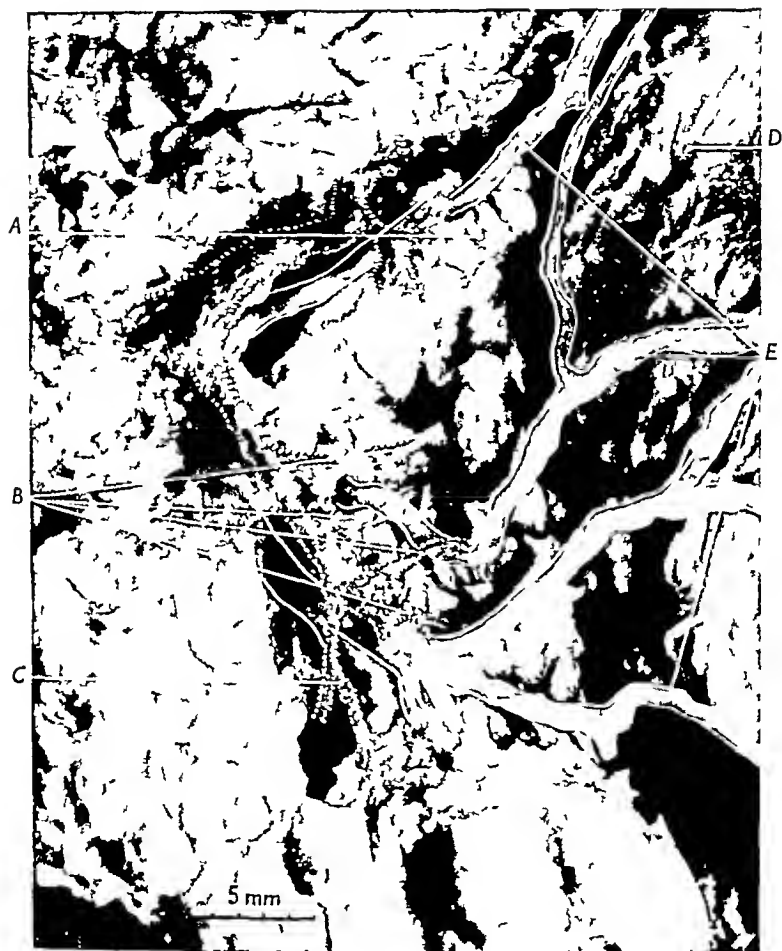
I am indebted to Prof R D Lockhart for his very helpful guidance and criticism throughout the investigation, to Mr A Cam for producing numerous photographs of the dissections, and to Mr W Chickshank for preparing the illustrations.

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EXPLANATION OF PLATE

Dissection of right parotid region showing masseter (*D*), and branches of the cervico-facial division of the facial nerve (*E*). (*A*) is a group of lobules clasping the upper branch of the cervico-facial division, while (*B*) indicates four ducts which are draining gland tissue deep to the nerves, passing between the nerves, and ending in the duct (*C*) which drains the superficial lobe.



THE POST-NATAL DEVELOPMENT OF RENAL TUBULES IN THE RAT

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Renal function in the newborn and very young differs in several ways from that in the adult. McCance & Young (1941) have shown that newborn infants always excrete a hypotonic urine, that their urea clearances are low as compared with adult values, and that clearances of sodium, chloride and potassium are likewise low, even when the plasma values are abnormally high. Gruenwald & Popper (1940) have pointed out that even the most mature renal corpuscles in the newborn have a thick glomerular membrane through which filtration must be difficult, and this would explain some of the differences. The low values for mineral clearances in the newborn suggest that the renal tubules, too, differ from those of the adult. Heller (1944) has brought forward confirmatory evidence of this, demonstrating that the renal tubules of newborn children react very poorly to the antidiuretic principle of the posterior lobe of the pituitary.

The vital dye, trypan blue, is stored by the cells of the proximal convoluted tubule of the kidney during its excretion (von Mollendorf, 1920), the greatest amount of dye being found in the tubule cells near the glomerulus and the intensity of the staining falling off as one passes distally along the tubule. The kidney of the rat at birth, and for some time afterwards, has a peripheral cortical zone of undifferentiated tissue, the neogenic zone (Felix, 1912), and we observed that the cells of this zone did not store trypan blue in very young rats stained *intravivam* with this dye. In contrast, the epithelium of the tubules in the deeper parts of the renal cortex contained dye droplets in their cytoplasm. It was considered that the ability to store vital dye must be related to functional differentiation of the nephron, and so it was determined to investigate the matter further to see if any light could be thrown on the structural basis of renal function in the very young individual and, in particular, on the differentiation of the neogenic zone of the cortex.

The majority of workers who have studied the storage of vital dye in the permanent kidneys of young animals have been interested in the question of renal function in pre-natal life. None of them appears to have worked upon the rat. The following summary of observations on the storage of trypan blue refers, then, to the metanephric tubules before birth in some mammals and birds, but certain points emerge which have some bearing on the results of the present work.

Wislocki (1920) injected trypan blue into the amniotic sac in guinea-pig foetuses and described and figured blue dye granules in the epithelial cells of the proximal convoluted tubules of the kidneys 24-72 hr later. Similar experiments

* Aided by a grant from the Colston Research Fund

were performed on cats, and like results were obtained (Wislocki, 1921*b*) when the injection was made directly into the peritoneal cavity of the foetus *in utero*

The reaction of the developing avian metanephros to intravital trypan blue has been studied by several workers. Wislocki (1921*a*) found traces of blue in the metanephric tubules of the 11-day chick embryo 48 hr after the injection of dye into the allantoic mesoblast. Hanan (1927) introduced dye into the air chamber of the egg and noted that it could be detected in the metanephros at 17 days of incubation. Hurd (1928), also working with the chick, investigated the matter in greater detail than the two previous writers. She found pale grey-green dioplets at 12 days in pyramidal cells of metanephric tubules with an indistinct lumen. These cells showed a thickened border with rows of cytoplasmic granules beneath it. At 16 days the dye-storing cells in the tubule wall were of two types, large and small, both having a definite 'brush' border. A similar series of observations was made by Sandstrom (1935) for the duck. He brought out a further point, namely, that the earlier metanephric tubules (19 days) stored the dye in the form of fine particles only, later, at 24 days, both fine and coarse masses were present. Sandstrom believed that when the dye was stored as fine particles only, the glomeruli and the secreting and collecting tubules were differentiated but not actively functional, when the cells of the proximal convoluted tubules showed dye in large masses active function occurred.

MATERIAL AND METHODS

The kidneys of 36 rats, ranging from newborn to 28 days old, were used in the present investigation. The animals had been stained intravital by a single intraperitoneal injection of trypan blue (0.5–1% aqueous solution) in a dosage of between 20 and 40 mg/100 g body weight. An interval of 3–6 days elapsed between the injection and the killing of an animal.

Rats stained intravital at younger than 3 days were obtained by intraperitoneal injection of trypan blue into the foetus *in utero*. Pregnant animals were anaesthetized with ether and the abdomen opened by a midline incision. The semi-transparent uterine horn was then gently manipulated with gauze pads soaked in warm saline until the belly of a foetus was clearly visible. A very fine hypodermic needle (no. 20), attached to a tuberculin syringe, was passed through the uterine wall and membranes until it perforated the anterior abdominal wall of the foetus. The desired amount of dye was injected, the needle withdrawn and the mother's abdomen closed. Very little tendency for dye to leak into the amniotic cavity was noted, and the fine puncture in the uterine wall sealed itself as soon as the needle was withdrawn. The operation did not interfere with the course of pregnancy and the animals came into labour at the normal time. One could not be certain just how much dye a foetus received in relation to its body weight, but well-stained healthy young were obtained with doses of 0.1–0.15 c.c. of a 1% solution injected 48 hr before the

end of gestation. On the other hand, in two cases the administration of 0.2 c.c. of the same solution caused necrotic changes in the kidney. Hanan (1927) noted a similar toxic effect when staining chick embryos *intra vitam* by repeated injections of trypan blue into the air chamber.

The histological methods used to demonstrate trypan blue in the renal epithelium were essentially the same as those used by one of us in a study of the *intra vitam*-stained adrenal (Baxter, 1946). While Bouin fixation, followed by staining with carmalum or safranin, gave the most brilliant pictures of dye droplets the quality of fixation was not always as good as could be desired, and several fixatives and staining methods were therefore used with each specimen.

OBSERVATIONS

Newborn rat. A well-stained animal was secured about 5 min. after birth. A sagittal section of the renal cortex (Pl 1, fig 1) shows a well-defined peripheral zone where the cells are embryonic in type and in which no trace of trypan blue is to be found. This is the neogenic zone. The inner two-thirds of the cortex is made up of glomeruli and tubules, and the epithelial cells of some of the latter contain blue droplets of vital dye. Such tubules may readily be identified by their histological characters as belonging to the proximal convoluted segment of the nephron. It may also be seen that the amount of dye and its depth of colour vary from tubule to tubule. The most intensely stained tubules are near the medulla; those underlying the neogenic zone show only a few small pale blue droplets in the epithelial cells.

If a proximal convoluted tubule be followed in serial sections it is found that the amount of dye in the epithelial cells is not constant throughout its length. The staining is heaviest at the neck of the tubule, it remains quite marked in the part distal to this and then diminishes gradually until none can be detected in the *pars recta* where the proximal tubule joins the descending limb of the loop of Henle. This gradient of dye storage along the course of the tubule has been noted by other workers (von Mollendorf, 1920, Cappell, 1929). The differentiated renal cortex of the newborn rat contains from within outwards, four or five rows of recognizable glomeruli and these differ in histological character according to their location (Pl 1, figs 9-11). The deepest ones are large and the glomerulus has a lobulated appearance. The more superficial nearer the neogenic zone are small and show no lobulation, the visceral layer of the capsule is thick. The intermediate glomeruli show gradations between these two forms. If comparable regions of the related proximal tubules be examined, e.g. the part just distal to the neck, differences are found in the number and nature of the dye droplets in the tubule cells. In the deepest tubules the droplets are large, of a deep blue colour and occupy much of the cell cytoplasm (Pl 1, fig 2). In tubules near the outer part of the cortex dye droplets are very few and quite tiny (Pl 1 fig 4) while the intervening ones (Pl 1 fig 3) show gradations between these extremes. The tubules associated with quite rudimentary glomeruli just deep to the neogenic zone exhibit no

recognizable dye in their epithelium. There appears, then, to be a differential reaction to trypan blue on the part of the nephrons of the newborn animal.

It is noteworthy that the cells of all the tubules storing vital dye have a 'brush' border on their luminal surface. This can be seen faintly in sections counterstained with carmalum, but is much clearer in control sections stained by the trichrome methods (Pl 1, figs 3, 9 and 10). The 'brush' border is small and ill-defined in tubules where there are few dye droplets; it is well marked in the deep tubules showing maximal storage of trypan blue, but is absent from the most superficial tubules beneath the neogenic zone in which likewise trypan blue cannot be detected.

Newborn to 12-day-old animals. During the first, and most of the second week after birth, nephrogenesis is a marked feature of the kidney in the rat. Precise numerical investigations (Kittelson, 1917) show that the number of nephrons at birth is more than doubled after 2 weeks. This results in an increase in the number of rows of glomeruli in the cortex to six or seven at 1 week, and seven or eight at 12 days. At the same time there is an increase in the number of tubules that show maximal dye storage. Thus, in the kidney of the 7½-day rat, the proximal tubules of the three deepest sets of nephrons in the cortex show many dense dye masses in their cell cytoplasm. The two sets of tubules superficial to this show the 'staircase' effect previously noted, while the tubules beneath the neogenic zone appear free from dye. At 12 days there are four layers of tubules showing maximal dye storage.

In the animals of this age group there are several cases where two rats of the same age have been stained intravital for the same time but with different doses of trypan blue. The significant differences between such pairs of animals are to be found only in the intensity of the staining. The pattern of dye storage in the cortex is the same whether the dose of dye be large or small if the time of exposure to it is constant; there is a comparable 'staircase' effect in the more peripheral proximal convoluted tubules but with the smaller dose the vital dye droplets are not so intense. The reasonable conclusion would appear to be that the developmentally older tubules store greater amounts of dye than those more recently differentiated.

About the eighth day following birth, and for some time afterwards, certain groups of cells in the neogenic zone become very evident because their cytoplasm contains much dye in dense blue masses. When followed in serial sections each group of these dye-containing cells immediately adjoins a differentiating glomerulus and runs in an arched fashion (Pl 1, fig 5) to the centre of a renal lobule (as defined by Traut, 1923). These tubular structures have not been observed to communicate with glomerular spaces. The epithelial cells forming them are low columnar in type, lack a 'brush' border, but have an astonishing avidity for trypan blue. In animals treated with small doses of dye for a relatively short time (24–48 hr), and in whom even the differentiated proximal tubules contain very moderate numbers of dye particles, these cells in the neogenic zone stand out vividly by reason of the brilliant blue masses

in their cytoplasm, rivalling in this respect the histiocytes of the perirenal connective tissue of the same animal. The wall of such a tubule segment is often irregular at one place and the lumen here contains pyknotic nuclei and debris.

Animals from 12 to 28 days of age. At 12 days some of the tubules just beneath the renal capsule show a few very fine, blue droplets in their cell cytoplasm (Pl 1, fig 6), and these cells also have a tiny 'brush' border on their luminal side. These are the proximal convoluted tubules of the most superficial layer of nephrons and they constitute the cortex corticis. In the tubule epithelium nearer the glomerulus the dye droplets are slightly larger and rather more numerous. On the other hand, nowhere in the most superficial layer of the renal cortex of the 10-day-old rat are there any dye droplets (except the coarse ones mentioned above), nor do the tubules show any sign of a 'brush' border. It would appear then, that final differentiation of the neogenic zone commences between these two stages.

With older animals the amount of dye storage in the cortex corticis increases (Pl 1, fig 7), and at the end of the fourth week it is maximal in all the layers of nephrons (Pl 1, fig 8). As the tubule epithelium becomes able to store more and more dye so the 'brush' border becomes larger and more differentiated.

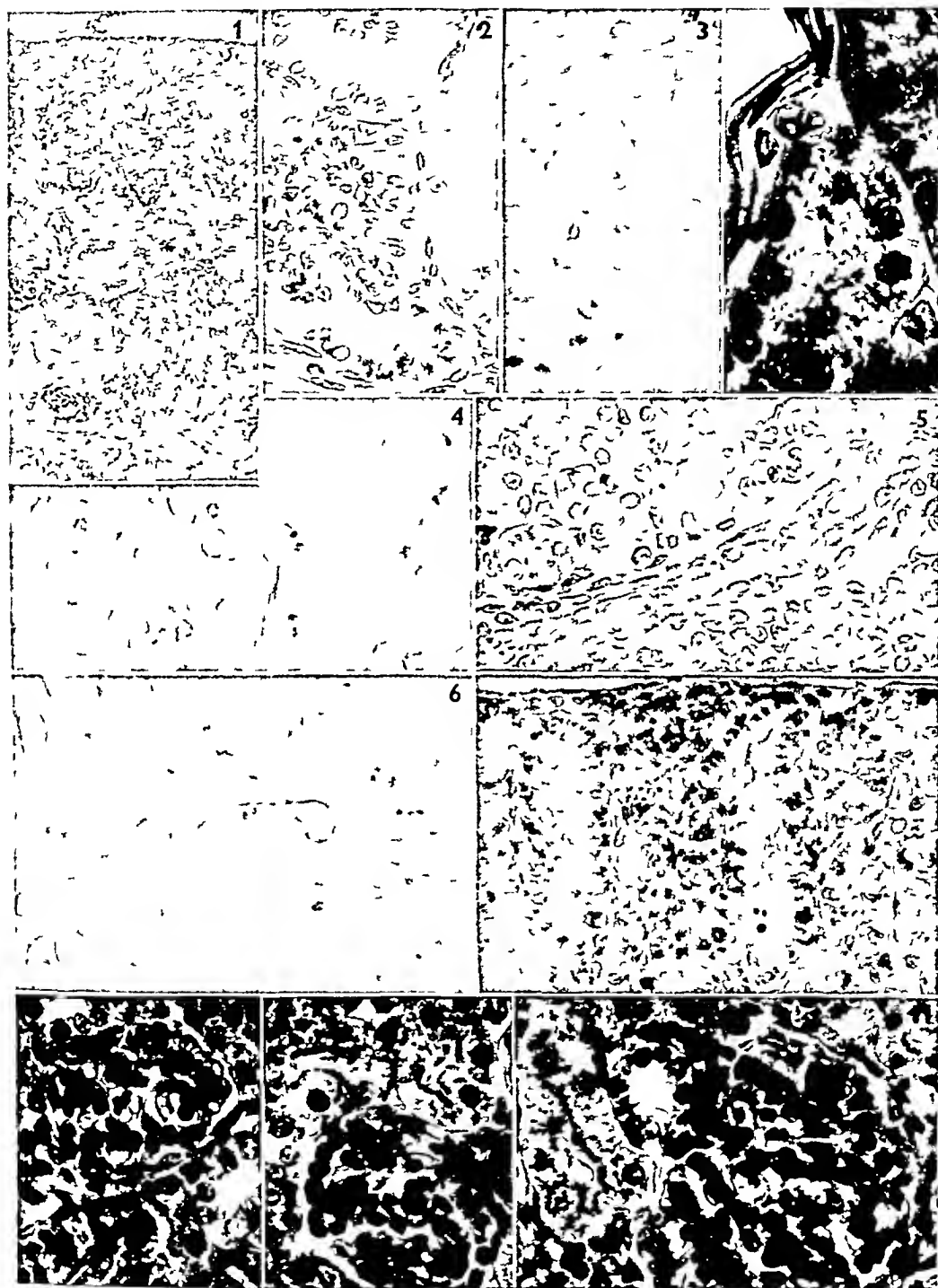
DISCUSSION

To assess the present results it is necessary to consider the route by which trypan blue reaches the tubular epithelium. On the one hand, it may pass from the intertubular capillaries and be concentrated in the cell cytoplasm, later to be excreted into the lumen of the tubule, or, on the other hand, it may be filtered through the glomerular membrane into the provisional urine, be re-absorbed into the epithelium from that fluid as it passes along the proximal convoluted tubule and be concentrated in the region of the Golgi apparatus in the cytoplasm. Although certain dyes (e.g. phenol red—Gersh, 1937) are known to be excreted into the tubule lumen by the first mechanism, the weight of available evidence and the present observations favour the view that trypan blue reaches the tubule cells from their luminal aspect (see von Mollendorf's review, 1920).

The question then arises: are not the differences in dye storage seen in the proximal tubules in newborn and young animals simply an expression of different amounts of dye in the provisional urine passing along these tubules? At first sight this would appear to be an attractive hypothesis. The three glomeruli of Pl 1, figs 9–11, from the same kidney, but themselves in different stages of development, show progressive thinning of the glomerular membrane with increasing lobulation between the capillary tufts as the glomerulus becomes mature. Filtration through the last one would be much easier than through the first. More provisional urine ought then to pass along the associated tubule whose epithelium would therefore have brought to it a greater total of dye in any given time, and so could store more. We are convinced, however, that this is not the complete explanation for differences in

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EXPLANATION OF PLATE

All the figures are from rats stained intravital with trypan blue except Figs 9-11

- Fig 1 Sagittal section of the renal cortex from a newborn rat. The neogenic zone (free from dye) occupies the upper part of the section, the medulla is below. Trypan blue is seen in varying amount in the epithelial cells of the differentiated proximal convoluted tubules. Carmalum stain $\times 100$
- Fig 2 Commencement of a proximal convoluted tubule in the deepest part of the renal cortex of a 2 day rat. Note the heavy dye storage in the epithelial cells. Carmalum stain $\times 410$
- Fig 3 Proximal convoluted tubule from the intermediate zone of the renal cortex of the same animal. Dye storage is not so marked as in Fig 3. Carmalum stain $\times 670$
- Fig 4 Another proximal convoluted tubule from the same kidney but situated nearer the periphery of the functional cortex. The blue dye droplets are small and few in number. Carmalum stain $\times 670$
- Fig 5 Neogenic zone of the renal cortex from a 10 day rat. Note the coarse dye droplets in the tubule running downwards and to the left towards the centre of the renal lobule. The surrounding tissue is quite free from dye. Carmalum stain $\times 410$
- Fig 6 Very fine trypan blue droplets in differentiating proximal convoluted tubules of the cortex corticis in a 12 day rat. The renal capsule is on the extreme left of the figure. Carmalum stain $\times 1140$
- Fig 7 Proximal convoluted tubule just deep to the renal capsule in a 16 day rat. Medium sized dye droplets can be seen in the cells. Haematoxylin ponceau fast green stain $\times 930$
- Fig 8 Peripheral renal cortex from a 28 day rat. The proximal convoluted tubules show approximately equal amounts of dye in their cell cytoplasm. Carmalum stain $\times 410$
- Fig 9-11 Immature, medium sized and mature glomeruli from the renal cortex of a 4 day rat. In Fig 9 note the tiny 'hrush' border on the cells of the proximal convoluted tubule just below the glomerulus. In Fig 10 the 'hrush' border on the associated tubule (above) is larger while in Fig 11 the proximal tubule leading from the glomerulus shows a fully differentiated 'hrush' border. Haematoxylin ponceau fast green stain $\times 500$

THE LYMPHATIC DRAINAGE OF THE SPINAL NERVE ROOTS IN THE RABBIT

BY E J FIELD AND J B BRIERLEY

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Attention has recently been drawn to the existence of a functional connexion between the spinal subarachnoid space and the prevertebral lymphatic system (Brierley & Field, 1948), though the detailed anatomical features of this communication could not be elucidated. The adoption of a new technique has now made it possible to outline completely the pathways in question and to make certain observations as to their character.

MATERIAL AND METHODS

The animal used was the rabbit, and all experiments were carried out on the living animal under nembutal anaesthesia. India ink, made by rubbing down a solid stick in physiological saline, was found to be unsuitable for direct introduction into the lymphatic system, since clumping of the particles prevented permeation of the finest vessels. Artificial stabilizing agents such as aerosol, fixanol, etc., were found to be worse than useless, as in many cases they actually brought about an immediate sedimentation of ink. The injection mass finally adopted utilized 4.5% reconstituted human plasma protein solution as dispersion medium, this being an average value for the protein content of lymph (Drinker & Yoffey, 1941). Such a suspension, filtered through a no. 5 Whatman paper, was made up fresh for each experiment. In it the range of particle size extended from 0.4 to 1.5 μ , but 90% were about 0.5 μ .

Three groups of experiments were carried out.

(1) In a preliminary series, ink was injected subcapsularly into the prevertebral and mesenteric lymph nodes. Such an injection gave good filling of the longitudinal channels on the front of the spine and of the small nodes along their course. The fine vessels leading dorsally from these channels were only filled, however, for 1 or 2 mm beyond the point at which they disappeared from view by passing under the opposed medial margins of the psoas muscles. In these experiments no obstruction was offered to the free return of lymph from the abdomen. The thoracic duct quickly became filled with ink, whilst no perceptible increase in resistance to the injection was encountered.

(2) In order to introduce an element of stasis in the abdominal lymph return and thus facilitate retrograde filling of the small vessels leading dorsally from the prevertebral nodes, another series of experiments was undertaken in which the multiple longitudinal lymph collecting trunks grouped round the aorta and inferior vena cava were isolated and individually ligated. Into one

of their number a fine cannula, directed caudally, was introduced. The cannula was connected to a reservoir of ink at a height of about 200 mm—a pressure well within the range recorded for this section of the lymphatic system (Drinkier & Yoffey, 1941). In only one of a dozen experiments was successful retrograde filling of the fine dorsally directed vessels obtained by this procedure (Pl 1, fig 1). The failures were due to the presence of valves in the larger vessels easily capable of withstanding the pressure employed. The successful case was the result of a lucky circumstance in which no valves were interposed between the site chosen for cannulation and the nearest dorsally directed fine segmental lymphatic.

(3) In order to dilate these vessels and thereby render their valves incompetent, it was decided to ligate the thoracic duct before introducing India ink into the posterior abdominal lymphatic system. Ligation of the duct in the neck proved an uncertain procedure, and the operation was therefore carried out in the thorax. Lymphatico-venous communications do not appear to have been reported in the rabbit, but should they be of the same type as in the monkey (Silvester, 1910) and rat (Job, 1918), then ligation of the thoracic duct would not be expected to produce more than a temporary stagnation of the abdominal lymphatic return.

A preliminary tracheotomy was performed, and respiration was maintained mechanically throughout the experiment. The animal was placed on its left side over a pad on an electrically heated operating table, the right paw pulled well forward, and an oblique incision made parallel to the vertebral border of the scapula. The latter was retracted and a convenient rib, usually the seventh, resected. In order to obtain an adequate exposure of the posterior mediastinum the posterior limit of the resection was placed well back in the sacrospinalis musculature. In the lateral position the rabbit is very sensitive to mediastinal displacement and so, as soon as the pleura was opened, aeration of the lungs was adjusted to reduce this as far as possible. A swab was inserted and the right lung gently pulled forward. The operation field was illuminated by the small bulb of an electric auriscope introduced into the chest. In some of the earlier experiments a preliminary feed of olive oil by stomach tube was found to be of help in the identification of the thoracic duct, but with practice this was found to be unnecessary. The duct was usually easily seen, either at once or after minimal dissection, immediately to the left of the vena azygos. On two occasions, however, it could not be identified with certainty and the operation then became a 'blind' one, the success or failure of which was checked post-mortem. A fine braided black silk suture on a small eyeless needle was passed round the duct and tied. Whilst this procedure entailed little danger to the aorta, the azygos vein was occasionally injured. Bleeding, however, invariably ceased when the ligature was tied. Successful ligation of the duct resulted in immediate distension of its distal segment. The chest was then closed with large interrupted catgut sutures as rapidly as possible. It was found to be important to turn the animal half on to the operated side as soon as feasible.

The expedition with which the chest could be closed and the animal turned into this position seemed to determine materially the success of the operation as measured by the number of hours survival.

Laparotomy was undertaken forthwith in all but seven cases. In these latter an interval of 20–30 min was allowed to elapse so that the general condition of the animal might improve before further operative trauma was inflicted. The prevertebral lymph nodes round the aortic bifurcation were cleared of overlying peritoneum and injected with India ink. The great lymphatic mass in the mesentery was similarly injected. In all cases injection was stopped as soon as resistance became at all appreciable. By repetition of this procedure at intervals of 2 or 3 hr as much as 4 or 5 c.c. of ink could be introduced, though excellent results were obtained with as little as 3 c.c. or even less. When a male animal was used, injection of the epididymis also was carried out. Altogether six out of twenty-two animals survived these formidable operative procedures for periods of 10–12 hr, during which time the pulse remained good and repeated doses of nembutal had to be administered to maintain an adequate depth of anaesthesia.

At the conclusion of an experiment the animal was perfused with saline followed by 10% formal saline. An adequate perfusion greatly facilitated post-mortem dissection.

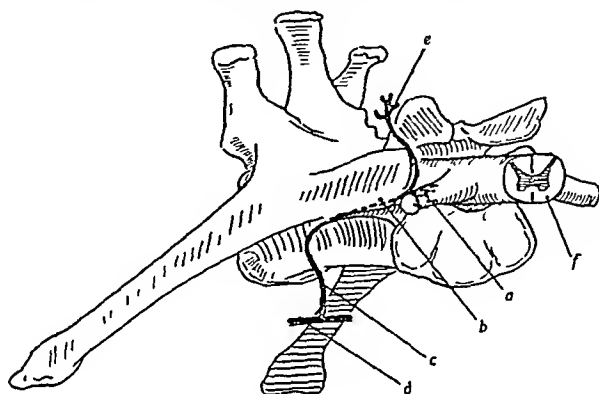
RESULTS

The full picture of retrograde flow of ink to the vicinity of the nerve roots was only met with in the six animals which survived operation for 10–12 hr. In those which were killed or died 2 or 3 hr after operation, ink was never found to have extended more than a millimetre or two backwards in the fine 'segmental' lymphatics. The lapse of 10–12 hr was essential to allow of backward movement of ink in the static lymph of these fine vessels.

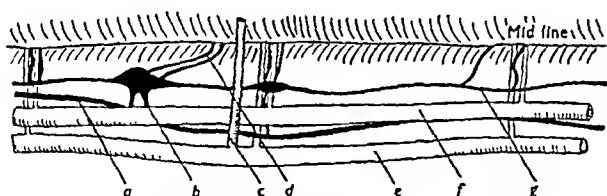
In the experiments of 10–12 hr duration, laminectomy revealed that in the upper lumbar and lower thoracic regions varying amounts of ink reached the vicinity of the dorsal root ganglia. In two of these six cases fine lymphatic vessels were found to commence in the substance of the erector spinae musculature where their radicles could be seen under the binocular dissecting microscope coursing between small bundles of muscle fibres. The vessels, two or three in number, passed ventrally in company with the blood and nerve supply of the muscle towards the region of the intervertebral foramen. Here tributaries came in from the neighbourhood of the dorsal root ganglion and its related epidural fat (Text-fig 1, Pl 1, fig 1). In these two cases India ink particles had actually reached the surface of the dorsal root ganglion and the adjoining cord membranes. The close relation between small ink-filled tributaries and the nerve just distal to the dorsal root ganglion is shown in Pl 1, fig 3. The lymphatics under consideration were wide channels lined with flattened endothelium (Pl 1, fig 4), and fine ink particles could be seen in all stages of passage through their walls. There was no swelling of the endothelial cells such as might have been expected if the particles had been actively phagocytosed. The

appearances suggested rather a purely passive movement of ink particles through the protoplasm of the endothelial cells. Outside the lymphatics many free ink particles could be seen lying in the meshes of the epidural fat of the intervertebral foramen.

Having received these tributaries from the dorsal root ganglion the main lymph vessel passed ventrally and cranialward in company with blood vessels closely opposed to the vertebral body under cover of a wing-like bony



Text fig 1 Lumbar vertebra and spinal cord from the left and behind (a) Nerve root with lymphatic radicles, (b) bony process hiding lymphatic afferents emerging from vertebral body (c) main lymph vessel joining longitudinal trunk (d) (e) afferent vessels from dorsal musculature, (f) spinal cord



Text fig 2 Dissection of the prevertebral lymphatic channels in the upper lumbar region. Two valveless longitudinal trunks (a, g) lie dorsal to the aorta and inferior vena cava (e, f) which have been displaced ventrally. Small prevertebral lymph nodules (b) are located along the trunks and from them fine dorsally directed vessels (d) pass between the opposed medial margins of the psoas muscles in the mid line. These are the vessels that constitute the segmental drainage of the spinal subarachnoid space, (c) left renal artery

plate (Text-fig 1 b). A fibrous sheet passed from the edge of the plate to the vertebral body to complete an osteofascial tunnel for the vessels. Whilst in this situation the lymphatic received a tributary from the interior of the vertebral body. After emerging from under cover of the medial edge of the psoas it ended in one of the longitudinal ducts related to the front of the spine (Text-fig 2). It was this terminal section of the vessel which had been noted previously as a 'segmental' lymphatic, following subarachnoid introduction of ink. Along

system which is the immediate factor in determining this outflow. Conditions which reduce this difference, either by lowering the subarachnoid pressure or raising the lymphatic pressure must therefore facilitate the diffusion of particulate matter against the centrifugal stream.

The lymphatic connexions of the spinal subarachnoid space must be taken into account in evaluating the clinical and experimental phenomena met with in poliomyelitis. The subject is an extensive one and has been reviewed elsewhere (Field & Brierley, 1948).

In addition to the tributaries from the dorsal root ganglion region the vessel which passes ventrally round the side of the vertebral body receives a tributary from its interior. This arrangement, should it turn out to be similar in man, may be of significance as affording an anatomical pathway by which secondary deposits may reach the spine from the abdominal viscera and from the testicle. Rouvière (1932) has shown the existence in man of lymphatic vessels arising in the sacrospinalis musculature and passing ventrally to the para-aortic nodes. These vessels are no doubt analogous to those here described, but the author gives no details of their course, nor does he mention any tributaries that they receive.

SUMMARY

1 The technique of ligation of the thoracic duct in the mid-chest region is described.

2 The anatomical detail of the lymphatic connexions between the nerve root region and the prevertebral lymph nodes is described.

3 The permeability of lymphatic endothelium to particles below 1.5μ diameter is confirmed.

4 The possibility of retrograde spread of infective agencies to the central nervous system along direct lymphatic channels is briefly discussed, and the conditions favourable to such an occurrence considered.

It is a pleasure to acknowledge the sustained interest and encouragement of Prof J M Yoffey. On general matters relating to the lymphatic system his never-failing advice and criticism have been invaluable. The authors are indebted to Dr G H Tovey, of the National Blood Transfusion Service, Southmead Hospital, Bristol, for the plasma used in their experiments, and to Messrs Abbott Laboratories for a supply of nembutal. Their thanks are due also to Dr D D Eley, University of Bristol, for his analysis of the factors involved in the diffusion of particulate matter. The histological preparations are the work of Mr Keith Hunt whose help the authors gladly acknowledge. All photomicrographs were taken on 35 mm film.

* Particulate matter is often used in biological problems as an indicator substance to determine fluid flow. Some knowledge of the physical factors which may influence the result obtained e.g. particle size, viscosity of medium, temperature, etc. is very desirable. Dr D D Eley of the Department of Chemistry, University of Bristol has kindly supplied the analysis set out as an appendix to this communication.

APPENDIX

DIFFUSION OF PARTICLES AGAINST A FLUID STREAM

Theoretical considerations

Consider first the flow of water down a capillary. The velocity v_w will vary from zero at the sides to a maximum at the middle—such a liquid shows parabolic flow

Consider a particle with a diffusion velocity v_D ($-v_D$ against stream). Its net velocity, v_p , will be the algebraic sum of v_D and v_w ,

$$v_p = v_D + v_w$$

For many purposes one might assume an average velocity across tube, v_w^A ,

$$v_p = v_D + v_w^A$$

For $v_p = 0$, zero net velocity,

$$-v_D = v_w^A - \text{the velocity of streaming}$$

The factors influencing v_D are determined by the Stokes-Einstein equation for the diffusion coefficient D , and we may assume $v_D \propto D$,

$$D = \frac{RT}{6\pi a \eta N},$$

N = Avogadro's number, T = absolute temperature, η = viscosity of liquid, R = gas constant, a = radius of particle

This equation holds for a *dilute* suspension of spherical particles of radius a , preferably considerably greater than the molecular radius of the liquid, i.e. it would hold for particles such as India ink or virus

We may then derive the conditions for a particle just not to diffuse against a certain fluid flow, v_w^A . It is

$$\frac{T}{a\eta} = \text{constant} \quad (1)$$

To appreciate the effect of temperature increase, we must allow for its effect on η . Then for water,

$$37^\circ \text{C} \quad \eta = 0.6947 \text{ centipoise}$$

$$38^\circ \text{C} \quad \eta = 0.6814 \text{ centipoise}$$

Thus a rise of 1°C affects T by only 1 part in 310 (310 – 311°K). η is affected by 1.3 parts in 69. In fact, for most purposes we express η as

$$\eta = \eta_0 e^{b/T},$$

where η_0 and b are constants for the liquid concerned. Then condition (1) becomes

$$\frac{T}{a\eta_0 e^{b/T}} = \text{constant}$$

Thus, treating the lymph as pure water to a first approximation, there is a logarithmic relation between a (the radius of particle which is just stopped by a certain average flow of fluid) and temperature, of the form

$$\log a = \text{constant} + \log T - \frac{b}{T}$$

Hence an increase in T will considerably increase the size of particle which will be able to diffuse against a constant fluid stream. Similarly, at constant temperature, any change in η of the lymph, due, say, to varying protein content, will affect inversely the limiting size of particle which will just be able to diffuse against a constant stream.

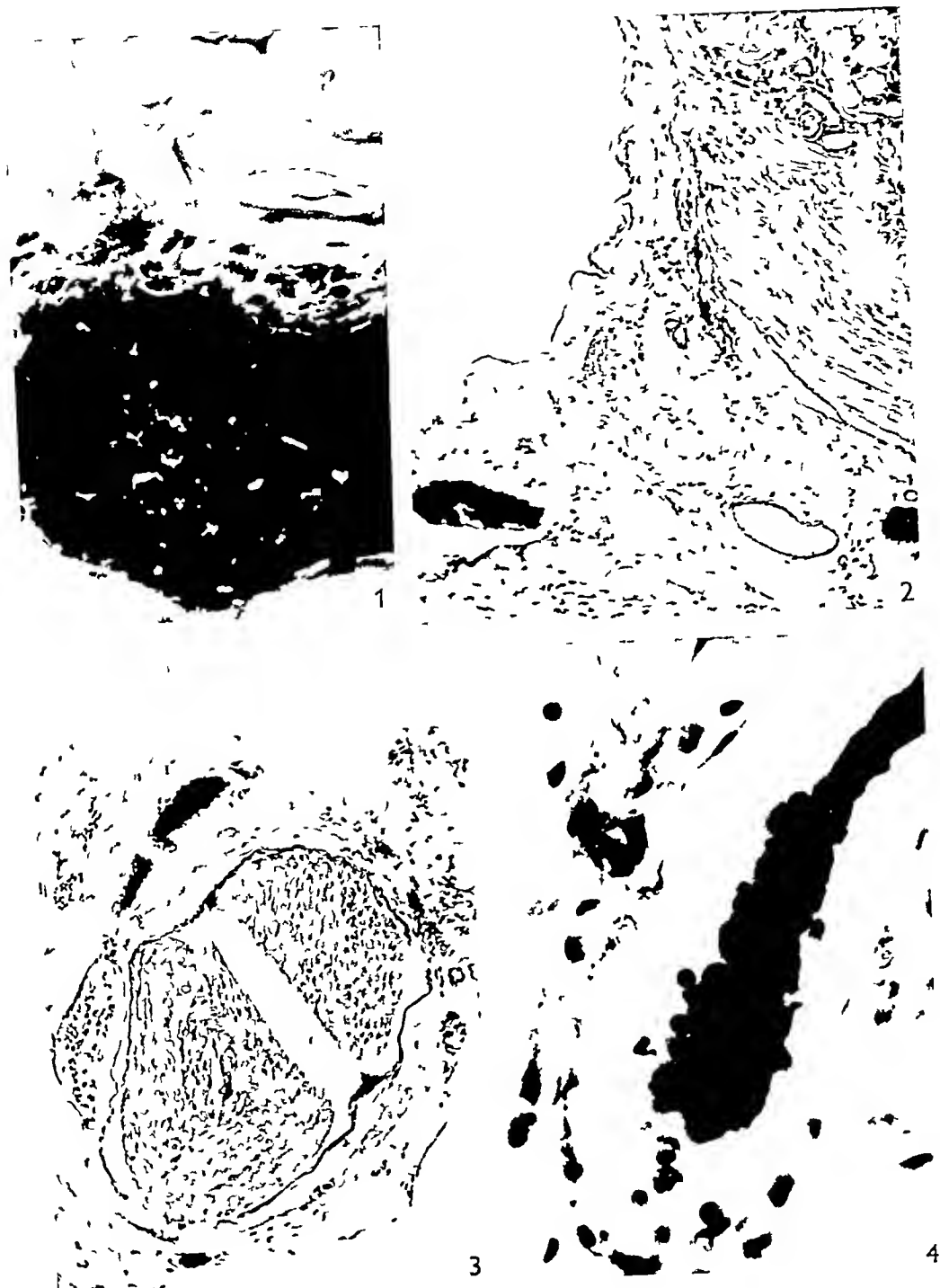
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EXPLANATION OF PLATE 1

- Fig 1 A lymphatic vessel filled with India ink, in close relation to the spinal nerve just beyond the dorsal root ganglion. Fine particles of free ink have migrated through the lymphatic wall and have come into intimate relationship with the nerve. $\times 1300$
- Fig 2 Ink filled lymphatics in the epidural fat close to the dorsal root ganglion. $\times 105$
- Fig 3 Lymphatics grouped round the nerve just distal to the dorsal root ganglion. $\times 105$
- Fig 4 Ink filled lymphatic close to dorsal root ganglion. The endothelial lining of the vessel is apparent and particles of India ink can be seen passing through it. The central dark mass is an accumulation of ink together with inflammatory cells. $\times 800$



CYCLIC CHANGES IN THE UTERINE MUCOSA OF BALAENOPTERID WHALES

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INTRODUCTION

This report presents the results of examining a series of specimens of uterine mucosa from antarctic whales. Very little work has appeared on this subject, a brief passage in the paper by Mackintosh & Wheeler (1929), which is referred to in the discussion below, containing the only observations on the changes in the uterine mucosa of whales that have been found in the literature. These authors examined a few specimens but investigated none from the later stages of pregnancy, thereby missing one of the most striking phenomena that occur in the cycle of changes.

In the present work the material is classified into groups according to the physiological state of the animal from which it comes, and the histological details characterizing each group are described. The cycle of changes that occurs during the progress from anoestrus through pregnancy and lactation back to anoestrus is outlined and the findings discussed in the light of the relevant literature. The material from pregnant whales here investigated consists of the mucosa from either the sterile cornu of the uterus or from the pregnant one or from both.

MATERIAL

The material consists of forty-four specimens of uterine mucosa from thirty-four whales, eighteen Blue, fifteen Fin and one Humpback. Data relating to date of capture, length, macroscopic appearance and width of the uterine cornua, number of corpora lutea in the ovaries, length of foetus if present, and thickness and state of the mammary glands are given as an Appendix. Each specimen consists of a block of tissue some 1-2 cm. cube from the wall of the cornu including a section through the mucosa and part or all of the muscular coats. No part of the chorion is present in any of the specimens from pregnant whales, it has presumably been stripped off, though no information on the point accompanies the specimens. The large chorionic sac with diffuse placenta fills both cornua in those Cetacea, several species of Odontoceti according to Mossman (1937), in which it is known. The foetus occupies the pregnant cornu, and the chorion within the sterile one is filled by the very large allantoic vesicle. There is no reason to doubt that the condition in the Balaenopterid whales differs materially, though no detailed description of the foetal membranes and placenta in these whales has yet appeared according to Mossman (1937). The material was fixed either in Bouin's fluid or in 10% formalin and was preserved in formalin. It is in fair histological state but some post-mortem

changes appear to have occurred in all specimens before fixation. This is only to be expected, because it is very difficult to obtain completely fresh material for histological purposes from whales. Decomposition starts very soon after death in these animals, insulated against heat loss by their blubber, and it is seldom that whales can be examined on a factory ship within an hour of their death. The material was collected in antarctic waters by Dr F. D. Ommanney on board the floating factory *Salvestria* during the southern whaling season of 1939-40, the earliest specimen being dated 14 December and the latest 28 February. The collection has been made available for examination by courtesy of Dr F. C. Fraser of the British Museum (Natural History) and of Dr N. A. Mackintosh of the *Discovery* investigations, to whom the author wishes to express his thanks.

The specimens from pregnant whales do not include the foetal placenta, which is diffuse, and being non-deciduous readily parts from the maternal tissues. The dimensions of the cornua in many of the whales were recorded in the field, the measurement made being the greatest width across the cornu in a collapsed state when lying on a flat surface. The corpora lutea present in the ovaries were noted in the field as 'corpora lutea A', young corpora lutea of ovulation or a current pregnancy, and 'corpora lutea B', old corpora lutea remaining from previous ovulations or pregnancies and partly or wholly transformed into corpora albicantia.

The mucosa from the specimens was examined in sections cut perpendicular to its surface at a thickness of 10μ . Sections from all specimens were stained with Heidenhain's haematoxylin and Beibich's scarlet, and further sections were stained with Mallory's triple stain.

In this paper all field measurements taken in feet and inches have been converted to the metric system.

CLASSIFICATION OF THE MATERIAL INTO GROUPS

A preliminary examination of the material after it had been sectioned showed that there is no important difference between the mucosa of the two closely related species, the Blue and Fin whales. The material from these two species has therefore been considered together in tracing the changes which occur before, during and after pregnancy, but the specimens from the single Hump-back whale are not included with these and are described separately.

The specimens fall naturally into five groups, those from whales which are immature, adult in anoestrus, ovulating, pregnant and lactating. The pregnant group is conveniently subdivided into three parts containing whales in early, middle and later pregnancy. The arbitrary standard here adopted for distinguishing the three is the length of the foetus: up to 0.762 m (2 ft. 6 in.) in the first, 0.762 m (2 ft. 6 in.) to 1.68 m (5 ft. 6 in.) in the second, and 1.68 m (5 ft. 6 in.) to 4.57 m (15 ft.) in the third. Table 1 shows the numbers and species in each of the seven groups thus defined.

TABLE I *Numbers and species of whales in each group*

Species	Immature	Anoestrus	Ovulating	Early pregnancy	Mid pregnancy	Later pregnancy	Lactating
Blue whale	2	3	—	1	3	6	3
Fin whale	1	3	1	4	1	5	—
Humpback	—	—	—	—	1	—	—
Totals	3	6	1	5	4+1	11	3

GENERAL NOTES ON THE WHALES IN EACH GROUP

Group α Immature

The whales in this group were all of adult size although they were sexually immature. The Fin whale was 19.35 m. and the Blue whales 21.54 and 22.61 m. long. In all of them the ovaries were immature, containing neither ripe follicles nor corpora lutea. The width of the uterine cornua ranged from 8.9 to 10.2 cm., and the mammary glands were virgin, measuring from 2.5 to 3.8 cm. in thickness.

Group β Adult in anoestrus

All the six whales in this group had been pregnant at least once. The three Fin whales ranged in length from 21.11 to 23.27 m., and the three Blue whales from 24.84 to 25.27 m. In none of them did the ovaries contain ripe follicles, but in all of them they contained one or more corpora albicantia. One Fin whale had been pregnant only once, for its ovaries contained a single corpus albicans. The ovaries of the other two Fin whales contained three and seven corpora albicantia respectively. One of the Blue whales had not been pregnant more than twice, the ovaries containing two corpora albicantia only, but the other two Blue whales were multiparous, their ovaries containing thirteen and sixteen corpora albicantia respectively. The width of the uterine cornua in the Fin whales ranged from 10.2 to 12.7 cm., in the Blue whales from 10.2 cm. in the whale with only two corpora albicantia to 78.8 cm. in the widest cornu of the whale with thirteen corpora albicantia. The smaller cornu of the latter whale was 40.6 cm. across, and a cornu of the whale with sixteen corpora albicantia was 20.3 cm. in width. No record of any high degree of vascularization in the uterus was made for any of these whales, and for two of them the field notes state that the vessels of the cornua were 'not congested'. In all the whales of this group the mammary glands were involuted and not virgin, showing that all including the youngest had not only been pregnant but had subsequently come into lactation. The thickness of the gland ranged from 2.5 to 10.8 cm. The differences in thickness of the involuted gland were probably due to the length of time since the last period of lactation rather than to the number of lactations that had occurred. This conclusion is supported by the fact that the gland was 6.4 cm. thick in the Fin whale which had been pregnant only once but only 2.5 cm. thick in that which had three corpora albicantia in the ovaries and 3.4 cm. thick in that which had seven corpora albicantia. Among the Blue whales the thickest mammary gland, which

measured 10.8 cm through, was in the whale with only two corpora albicantia, and the thinnest measuring only 5.1 cm through was in the whale with sixteen corpora albicantia

Group γ Ovulating

Only one whale, a Fin whale 22.12 m long, falls into this group. The ovaries contained three recently formed corpora lutea and no less than forty corpora albicantia. It has generally been held that whales have a single ovulation at each oestrus, and that in the absence of fertilization there is a succession of short polyoestrous cycles. The three fresh corpora lutea in the ovaries show that the whale was experiencing such a succession of cycles, and the very large number of corpora albicantia suggests that this animal had experienced similar infertile polyoestrous cycles in the past. No embryo was found in the uterus although search was made. Ovulation had very recently occurred, and it is likely that the blastocyst if present had not yet reached the uterus, ova or blastocysts in the uterine tube are very easily missed during the examination of whales that are being utilized commercially. This whale had been pregnant and had lactated previously, for the mammary gland was involuted and 5.1 cm in thickness.

Group δ Whales in early pregnancy

This group comprises the five whales containing foetuses up to but not exceeding 0.762 m (2 ft 6 in) in length. Four of them were Fin whales ranging from 18.49 to 22.35 m in length, the remaining Blue whale being 22.76 m long. The smallest Fin whale was in its first pregnancy, the ovaries containing only one corpus luteum and the uterus a foetus 0.242 m long, the pregnant cornu being 24.2 cm in width. The mammary gland was only 2.5 cm in thickness and was virgin, showing that lactation had never occurred. The Blue whale was also probably in its first pregnancy, as shown by the virgin state of the mammary gland which was 3.1 cm thick. But the ovaries contained the corpus luteum of the recent ovulation and a corpus albicans. The latter probably represented a sterile first oestrus but might have represented an abortive pregnancy. The width of the uterine cornu on the side of the ovary with the young corpus luteum was 20.3 cm, that of the other 22.9 cm. No foetus was found in this whale but the field note observes 'very small foetus missed?'

The remaining three Fin whales had all been pregnant previously, as shown by their involuted mammary glands which ranged in thickness from 1.4 to 7.0 cm. In each the ovaries contained one large young corpus luteum of the current pregnancy and respectively three, six and nine corpora albicantia. Two contained foetuses, but the field note for the third states that no foetus was found though both cornua were searched. In the latter whale the uterine cornu of the side corresponding to the ovary containing a young corpus luteum and two corpora albicantia measured 20.3 cm across and the other cornu, whose corresponding ovary contained one corpus albicans, 17.8 cm. Both were

noted as being 'not congested'. One of the other whales contained a male foetus 0.242 m long in a cornu 24.2 cm wide, the sterile cornu being 25.4 cm wide. The ovaries contained one young corpus luteum of pregnancy and nine corpora albicantia. The third whale contained a male foetus 0.381 m long, no data are recorded about the dimensions of the uterine cornua, but the ovaries contained one corpus luteum and six corpora albicantia.

Group ε Whales in mid-pregnancy

Four whales, three Blue and one Fin, fall into this group. The lengths of their foetuses ranged from 0.94 to 1.51 m, and two of the mothers were in their first pregnancies.

The Fin whale, 19.94 m long, contained a male foetus 1.32 m long, and the width of its pregnant cornu was 55.9 cm. The ovaries contained only one corpus luteum, a young one, and the virgin mammary gland was 3.1 cm thick. One of the Blue whales, 23.11 m long, was also in its first pregnancy, and contained a female foetus 1.45 m long. The pregnant cornu was 73.7 cm wide and the sterile one 49.4 cm. The ovaries contained only one corpus luteum, that of the current pregnancy, in the ovary corresponding with the pregnant cornu. The mammary gland was in the virgin state and measured 3.8 cm in thickness.

Both the other whales in this group are Blue whales and had been pregnant previously as shown by the involuted state of the mammary glands. One, whose length is not recorded, contained a male foetus 0.94 m long, and the width of its pregnant cornu was 81.3 cm, of its sterile one 50.8 cm. The ovaries contained one young functional corpus luteum and five corpora albicantia. The involuted mammary gland was 5.1 cm in thickness. The other whale, 27.00 m long, contained a female foetus 1.51 m long. The measurements of the uterine cornua are not recorded, but the field note states that the cornua were 'not congested'. The ovaries contained one corpus luteum and ten corpora albicantia. The involuted mammary gland was 8.2 cm thick.

Group ζ Whales in later pregnancy

The length of the foetuses in the whales of this group ranges from 1.73 to 4.50 m. Eleven whales come under this category, five Fin and six Blue, and all had been pregnant at least once before, the mammary glands of all of them being involuted and not virgin. In Table 2 are summarized the data relating to these whales, their foetuses, uterine cornua and mammary glands.

It will be noted from these data that one whale, SV204, was in its second pregnancy, and another, SV46, in its second or third. All the others had probably been pregnant more than once previously, some of them many times, though the high numbers of corpora albicantia in SV40 with twenty-two and SV48 with thirty-five do not necessarily imply that a corresponding number of pregnancies had been experienced. These high numbers merely show that the whales were comparatively old and multiparous.

TABLE 2 *Data from whales in later pregnancy*

Whale no	Species	Date	Length (m)	Field notes and width of cornua (cm)		Corpora		Foetus		Mammary gland thickness (cm) (all involved)
				Pregnant	Sterile	albica taria	albica taria	Length (m)	Sex	
SV40	Fin	19 XII	21.67	—	—	1	22	1.73	F	5.7
SV263	Blue	25 I	26.14	78.8	—	1	16	1.85	F	11.5
SV274	Fin	26 I	21.2	67.4	48.3	1	15	1.89	F	6.4
SV204	Fin	18 I	18.52	—	—	1	1	1.91	M	5.1
SV44	Blue	20 XII	27.13	'Not very congested'		1	11	2.01	M	6.4
SV46	Blue	22 XII	24.79	83.9	—	1	2	2.59	M	3.8
SV94	Blue	31 XII	26.59	—	—	1	13	2.82	F	10.2
SV490	Fin	24 II	21.41	96.5	—	1	5	3.10	F	3.2
SV32	Blue	18 XII	23.85	—	—	1	6	3.08	F	4.4
SV48	Blue	24 XII	26.95	139.7	62.3	1	35	3.89	F	10.2
				'Both heavily congested'						
SV416	Fin	15 II	22.10	77.5	—	1	6	4.50	F	1.4

Group η Lactating

Three Blue whales form this group, one of them lactating for the first time, another for not more than the second time. The first was lactating freely and contained one corpus albicans. It was 24.41 m long, the uterine cornua both measured 11.5 cm across and the mammary gland was 27.0 cm thick. In the whale 23.17 m long, probably lactating for the second time, the ovaries contained two corpora albicantia, and the uterine cornua were both 16.5 cm wide and noted as 'not congested'. The thickness of the mammary gland was not measured, but the whale was recorded as 'lactating slightly'. The third whale was 22.45 m long and was an old multipara as shown by the presence of nineteen corpora albicantia in the ovaries. The uterine cornua were 22.9 and 25.4 cm wide respectively, and the freely lactating mammary gland was 24.2 cm thick.

HISTOLOGICAL STRUCTURE OF THE MUCOSA IN
EACH GROUP

Before considering the changes that are observed in the mucosa in passing from one group to another, a description is now given of the histology of the mucosa in representative examples from each group.

In all specimens the mucosa is clearly divisible into two layers, a thinner subepithelial layer, the stratum compactum, containing only the ducts of the glands, and a thicker deep layer, the stratum spongiosum, containing the convoluted parts of the glands.

Group α Immature

Among the three whales in this group the thickness of the mucosa as a whole ranges from 0.5 to 1.5 mm (mean 1.2 mm), and of the stratum compactum from 80 to 200 μ (mean 112 μ). The surface of the mucosa is smooth or divided by only a few minute furrows. In all specimens the epithelium has been lost

from the surface of the mucosa, probably post-mortem (Pl 1, fig 1) In SV47 the glands are few, scattered and not greatly convoluted. They are 30–40 μ in diameter, have small or no visible lumina, and are lined by columnar epithelium. The nuclei of the gland epithelium fill the greater part of the cells in the deeper part of the stratum spongiosum, but occupy only about the basal halves of the cells in the parts of the glands immediately below the stratum compactum. The ducts in the latter stratum are few and widely scattered, they are lined by a similar columnar epithelium having the nuclei at the bases of the cells. This epithelium was presumably continuous with that lining the mucosa, but in the specimen it stops short at the mouths of the glands, the epithelial lining of the uterine lumen having been lost apparently as a result of post-mortem changes before fixation. The capillaries of the mucosa appear to be slightly more numerous than the glands, but larger vessels are few. At the base of the mucosa and in the submucosa the vessels are much larger and more conspicuous. The submucosa is about equal in depth to the mucosa and consists of a very loose areolar and connective tissue among which lie the larger blood vessels.

The stratum compactum consists of a closely packed connective tissue seven or eight cells thick. Its outer layers are rather flattened and tend to be stratified. The layer of flattened cells is three to four cells thick, and the deeper cells beneath it are polyhedral. The closely packed cells of the stratum compactum are small so that the nuclei fill a comparatively large proportion of the cell volumes. This concentration of nuclei gives a characteristic pattern to the stratum compactum in contrast to the much more loosely arranged stratum spongiosum with its more widely scattered nuclei. In addition the stratum compactum contains less collagenous material, so that the nuclei stand out conspicuously against a paler background in specimens stained with Mallory's triple stain.

In SV280 (Pl 1, fig 2) the surface of the mucosa is interrupted by more numerous and conspicuous depressions at the bases of which the mouths of the glands open. The glands are larger, some of the more superficial ones having a well-marked lumen. The stratum compactum is thicker and the cells in it are less concentrated.

Group β Adult in anoestrus

In this group the thickness of the mucosa ranges from 1.5 to 4.0 mm, the mean being 2.4 mm. The thickness of the stratum compactum ranges from 50 to 400 μ (mean 194 μ). The vessels are much larger and more numerous than in specimens from the previous group because all the whales had been pregnant at least once.

In SV283 (Pl 1, fig 3) the surface of the mucosa in the larger cornu is smooth and raised into low ridges. The glands are numerous and much convoluted, 40–50 μ in diameter, and have visible though small lumina. They are lined with columnar epithelium whose large nuclei fill the basal half to two-thirds of the cells. In the deeper parts of the mucosa the nuclei of the gland

cells are approximately circular and fill only the basal halves of the cells, in the more superficial parts the nuclei are oval, the axes of their long diameters coinciding with those of the cells, and they occupy fully the basal two-thirds of the cells. Ducts from the glands, penetrating the stratum compactum to open at the surface, are very few. Vessels, many of them with thickened walls and reduced lumina, are very numerous throughout all parts of the stratum spongiosum, and are very large, tortuous and numerous in the submucosa. The surface of the stratum compactum, as in group α , is rendered conspicuous by the closely packed nuclei, and its superficial cells tend to be stratified. Vessels much smaller in size than those of the stratum spongiosum are numerous in the stratum compactum. In the stratum spongiosum the connective tissue tends to be concentrated round the glands, between them it is loose. Where shrinkage has occurred during fixation the looser tissue between the glands has split. The surface of the mucosa is without an epithelium as in all specimens in this group.

Group γ Ovulating

One whale only, SV102, forms this group. The thickness of the mucosa ranges from 7.0 to 10.0 mm, the mean being 8.5 mm, and of the stratum compactum from 150 to 500 μ , the mean being 313 μ . The surface of the mucosa is in general smooth (Pl 2, fig 5), but in many places it is finely wrinkled (Pl 1, fig 4). In the deeper parts of the stratum spongiosum the glands are numerous, closely packed and greatly convoluted, they are from 60 to 100 μ in diameter. They are mostly without lumina and are filled with a mass of polyhedral cells, the outermost ones in places being columnar and having their nuclei at about the middle of their long diameters so that clear cytoplasm fills them at their inner and outer ends. In the more superficial parts of the stratum spongiosum the glands are less closely packed, smaller in diameter and have clear lumina. Here the epithelial lining is columnar, with large nuclei filling the greater part of the cells. The convolutions of these parts of the glands are much less complex than in the deeper parts, and in places they are absent so that small lengths of straight duct are formed. Few ducts can be seen actually opening on to the surface of the mucosa. Large tortuous vessels are numerous at all levels of the stratum spongiosum, and are no larger or more plentiful in the submucosa. The surface of the stratum compactum is conspicuous from its more closely packed nuclei and absence of glands, only a few ducts traversing it. Its most striking feature, however, is a close network of capillaries engorged with blood corpuscles at its extreme outer surface, with no tissue other than the walls of the capillaries intervening between the lumina of the capillaries and the uterus (Pl 2, figs 6, 7). Many of the capillaries are so superficially placed that they bulge into the uterine lumen and appear as if applied to the surface of the mucosa. The capillary walls are everywhere intact and nowhere is there any sign of spontaneous rupture. Slightly larger vessels run through the stratum compactum to supply the superficial capillaries, but they are not

numerous. The majority of the cells of the stratum compactum have fusiform nuclei and tend to be arranged in a stratified manner with the long axes of the nuclei parallel to the surface of the mucosa. There is no epithelium bounding the surface of the mucosa. The connective tissue of the stratum spongiosum is extremely loose, the cells are concentrated round the glands and the spaces between them are occupied by scattered cells and fibres, from this condition the inference is drawn that the mucosa was oedematous.

Group 8 Whales in early pregnancy

The mucosa ranges in thickness from 1.0 to 11.0 mm, the mean being 4.0 mm, the stratum compactum from 130 to 580 μ , the mean being 295 μ . There is a considerable range of variation in the condition of the mucosa and glands. Two examples selected from this group are therefore described.

In the mucosa of the pregnant cornu of SV 89 (Pl. 2, fig. 8) the glands are closely packed and measure from 80 to 100 μ in diameter. Most of them are entirely filled with a loose mass of cells, a condition perhaps due to post-mortem histolysis, for the cells bounding the lumen are for the greater part columnar and in many places show a tendency to break away into it. The parts of the glands occupying a layer about 500 μ thick immediately beneath the stratum compactum differ conspicuously. They are very closely packed, leaving little connective tissue between adjacent glands, and are larger in size measuring 100–120 μ in diameter. Their lumina, too, are lined with very tall columnar cells and filled with a mass of loosely packed cells which appear to be columnar cells displaced by post-mortem changes. Large thin-walled vessels are numerous throughout the stratum spongiosum and in the submucosa. Very large vessels are also present among the layers of the muscular coats. The surface of the stratum compactum is smooth, and in transverse section appears as deeply incised where, here and there, incipient crypts are in course of formation (Pl. 2, fig. 9). The boundaries of the lumina of the incipient crypts passing through the stratum compactum are uneven, presenting an irregularly serrate outline in both transverse and longitudinal section. The crypts follow tortuous courses, so that in sections vertical to the surface of the mucosa they are seen to be cut transversely, obliquely and longitudinally. The cells of the stratum compactum are concentrated in the superficial layer and round the crypts. Many capillaries lie on the surface of the mucosa, on which there is no epithelium.

In the pregnant cornu of SV 472 (Pl. 3, fig. 10) the glands are less closely packed and are embedded in a rather loose connective tissue stroma, they are 50–80 μ in diameter. They are lined with tall columnar epithelium and have small or no lumina. Immediately below the stratum compactum they are larger, from 80 to 160 μ in diameter, lined with a similar epithelium and have conspicuous lumina. Vessels both large and small are numerous in the stratum spongiosum. The surface of the stratum compactum is deeply cut up by narrow depressions which are crypts in the course of formation. Many of these depressions are branched and irregular in outline, so that the tissues inter-

vening between are seen as irregularly lobulated processes. Small vessels are fairly abundant in the stratum compactum, the surface of which is covered with a network of capillaries extending to the bottom of the depressions. The capillaries, everywhere intact, bulge above the surface of the mucosa into the uterine lumen. The cells in the superficial parts of the stratum compactum are more closely packed than in the deeper ones. There is no surface epithelium.

Group e. Whales in mid-pregnancy

The mucosa ranges from 2.0 to 7.0 mm in thickness, the mean being 3.4 mm, and the stratum compactum from 90 to 700 μ , with a mean of 255 μ . The surface of the mucosa is much cut up by the openings of the crypts, so that irregularly lobulated processes are left between them.

In the pregnant cornu of SV164 (Pl. 3, fig. 11) the glands in the deeper parts of the stratum spongiosum are closely packed and from 40 to 80 μ in diameter, the mean being 60 μ . They have small lumina lined with tall columnar epithelium, but in many places the lumina are filled with loose cells and the epithelium is irregular, apparently as a result of post-mortem changes. There are some large vessels and numerous small ones in this part of the mucosa. In the part of the stratum spongiosum immediately underlying the stratum compactum the glands are much larger, measuring 60–170 μ in diameter and having large lumina lined with tall columnar cells. Many of the lumina contain some cellular debris. Comparatively large vessels as well as small ones are numerous at this level. The stratum compactum is formed of rather loose connective tissue slightly more condensed just beneath its surface. At its base, immediately superficial to the large glands of the stratum spongiosum just described, the gland ducts, 200 μ and more in diameter, pursue a tortuous course, here and there passing through the thickness of the stratum compactum to open into the uterine lumen at the bottom of a crypt. The ducts are lined with columnar epithelium which has in most places become detached from the wall so that the cells lie disordered in the lumina of the ducts. The crypts are irregular in outline both in longitudinal and transverse section, and the tissue between has proliferated so that they are separated by lobulated and branching processes of some complexity. There are many vessels of medium size in the stratum compactum, and a number of very conspicuous large ones with thin walls which extend in places quite to the surface. The whole of the outer surface of the mucosa, including the ramifications of the branched processes, is covered with a superficial network of capillaries (Pl. 3, fig. 12). Similar capillaries in rather smaller quantity surround the ducts in the deeper layers of the stratum compactum and lie immediately below the columnar epithelium. The surface of the mucosa bounding the uterine lumen is without epithelium. The conditions in the sterile cornu of SV247 (Pl. 3, fig. 13) are very similar.

Group ζ Whales in later pregnancy

The mucosa ranges in thickness from 1.5 to 8 mm, the mean being 3.7 mm, and the stratum compactum from 200 to 1000 μ , the mean being 263 μ . The most conspicuous character of the mucosa in this group is the high degree of complexity attained by the branching processes of the surface between the crypts. In transverse section of the mucosa these processes appear in some examples as extremely complicated dendritic structures. Several specimens from this group will be described because there is considerable diversity in the conditions found, ranging from those little altered from the state of the mucosa in the last group to the greatest degree of complexity of the dendritic processes and a high degree of activity of the glands.

In the pregnant cornu of SV44 (Pl. 3, fig. 14) the glands of the deeper part of the stratum spongiosum are from 40 to 60 μ in diameter and are completely filled with cells. The outermost cells form a layer of columnar epithelium, and the cells filling the lumen are probably debris resulting from post-mortem changes. In the superficial part of the stratum spongiosum immediately below the stratum compactum the glands are larger, from 80 to 120 μ in diameter, and are lined by a very tall columnar epithelium with the nuclei towards the bases of the cells. The small lumina are filled with loose cells. Vessels are numerous throughout the mucosa, but are largest in the stratum compactum and the immediately subjacent part of the stratum spongiosum. The ducts of the glands follow a tortuous course through the stratum compactum and open into the crypts between the lobulated processes extending into the uterine lumen as in the last group. The connective tissue cells of the stratum compactum are concentrated in the layer immediately below the free surface and around the ducts. The free surface of the mucosa and the lumina of the ducts are covered by a network of capillaries. Here and there traces of a cubical to flattened epithelium remain on the surface of the mucosa between the branched processes.

In the sterile cornu of SV46 (Pl. 4, fig. 15) the glands are very closely packed throughout the stratum spongiosum. In the deeper parts they are 40–60 μ in diameter and have conspicuous lumina. They are lined by columnar epithelium with the nuclei at the bases of the cells. More superficially the glands are 60–80 μ or more in diameter and have small lumina, because the epithelium consists of very tall columnar cells with nuclei about one-third of the distance from the bases. The surface of the mucosa is covered with dendritic processes of very complex outline. The processes are derived not only by proliferation of the tissue forming them, but also by the enlargement and coalescence of the crypts cutting down into the stratum compactum. As a consequence the stratum compactum apart from the dendritic processes is greatly reduced in thickness, the glands at the limit of the stratum compactum abutting almost directly on the bases of the processes and the bottoms of the crypts between them. A number of vessels larger than those of the stratum spongiosum occur

in the stratum compactum and extend into the processes. The surface of the processes is covered with a network of capillaries which is clothed in places with patches of thin epithelium.

In the pregnant cornu of SV40 (Pl 4, fig 16) the glands all have conspicuous lumina, the larger ones filled with a coagulum. The deeper glands are $60-80\mu$ in diameter, the most superficial ones $60-200\mu$. All are lined by columnar epithelium which is nowhere particularly tall. There are some very large vessels in the stratum spongiosum and many smaller ones in the stratum compactum and in the dendritic processes. The processes are partly covered by a flattened epithelium beneath and between the cells of which the capillary network ramifies. Some of the processes are invaded by large numbers of leucocytes.

In the sterile cornu of SV48 (Pl 4, fig 17) all the glands have conspicuous lumina, the deeper ones being $80-100\mu$ in diameter, the more superficial ones $100-250\mu$. They are lined with columnar cells with the nuclei near the bases, the cells of the more superficial glands being taller than those of the deeper. The glands are closely packed, there being little connective tissue between them. In this specimen the dendritic processes reach their greatest complexity, branching and rebranching many times to form a mass of small twigs. With this great proliferation of the processes the crypts become so deep that the stratum compactum is practically non-existent as a separate layer beneath them. The main trunks of the processes contain comparatively large vessels, but the smaller twigs consist of a tenuous core of connective tissue supporting a close network of capillaries which is covered in places by a thin epithelium. The capillaries and vessels with their contained blood form by far the greater part of the bulk of all but the main trunks of the processes.

Group η Lactating

In this group the mucosa ranges from 0.5 to 4.0 mm, the mean being 2.1 mm, and the stratum compactum from 50 to 250μ , with a mean of 163μ . The dendritic processes are reduced or completely absent, and the crypts remain as only a few comparatively shallow depressions interrupting the smooth surface of the mucosa.

SV245 was lactating freely, and the following conditions were found in the cornu on the side opposite to the ovary with the largest corpus albicans, presumably the one that had been sterile (Pl 4, fig 18). The glands of the deeper parts of the mucosa are $40-50\mu$ in diameter, and in a narrow superficial layer immediately beneath the stratum compactum $50-80\mu$. They are lined by narrow columnar cells and have very small or no lumina. The stratum spongiosum contains many very large thick-walled vessels which follow tortuous courses through it. The stratum compactum contains a number of similar though smaller vessels. The connective tissue cells of this layer are concentrated immediately beneath the free surface, and at intervals this concentration of cells extends downwards into the stratum compactum as a strand. These strands give the appearance of having been formed by the closure of the crypts.

between the bases of the formerly existing dendritic processes. The sides of the crypts appear to come into apposition and to fuse, the resulting band of closely packed cells being subsequently withdrawn to the surface. There is no superficial network of capillaries, and the surface is in places clothed with a thin cubical or flattened epithelium.

SV 127 was lactating slightly, and in the presumed sterile side the following conditions were found (Pl. 4, fig. 19). Involution has gone further than in the last specimen, though the surface of the mucosa still possesses large remains of the dendritic processes. The large vessels with thick walls are reduced in size, and the glands are few and small, being only from 20 to 40 μ in diameter with no visible lumina. The glands of the superficial part of the stratum spongiosum are not noticeably larger than those of the deeper part. The stratum compactum contains few vessels, and the cells are concentrated near the surface with no overlying capillary network. There are in places patches of low cubical epithelium on the surface of the mucosa.

The Humpback Whale

The single Humpback whale 14.45 m. long corresponded with the mid-pregnant group of Fin and Blue whales, although it contained a male foetus only 0.534 m. long. Owing to the smaller size of this species the division of the stages of pregnancy would obviously need to be based on a different scale of foetus length from that used for the larger species. The ovaries contained one corpus luteum and four corpora albicantia, the mammary gland was 7.0 cm. thick and involuted.

The mucosa of the pregnant cornu is 10.0–11.0 mm. thick, and the surface is already covered with small dendritic processes. The stratum compactum is 250–300 μ thick, and contains a number of irregularly shaped incipient crypts that are beginning to produce the dendritic processes by cutting up the stratum compactum. The glands of the superficial part of the stratum spongiosum are 50–120 μ in diameter, closely packed and lined by tall columnar cells, their lumina are almost entirely filled with loose cells. In the deeper part of the stratum spongiosum the glands are 30–50 μ in diameter, lined with columnar cells, and have small lumina. Large vessels are plentiful in the deeper parts of the stratum spongiosum and less numerous in the more superficial parts where, however, they occur right up to the boundary of the stratum compactum.

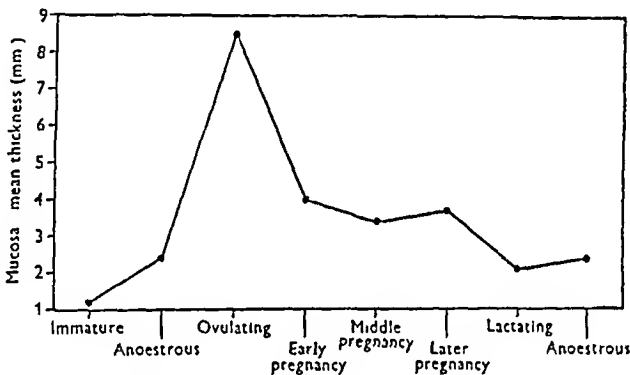
In the non-pregnant cornu the mucosa is 5.0 mm. thick and the surface is much smoother, the processes being fewer, smaller and sparsely lobulated but not dendritic. The stratum compactum is 200–300 μ thick and contains few ducts. The glands of the superficial and deeper layers of the stratum spongiosum are not conspicuously different from each other. In both layers their diameters range from 40 to 50 μ , they are lined with small columnar cells and have small lumina. All the glands of this cornu resemble those of the deeper parts only of the stratum spongiosum in the pregnant cornu, there being no enlarged

superficial ones. The vessels of the stratum spongiosum are neither large nor numerous.

THE CYCLE OF CHANGES IN THE MUCOSA

The changes in the uterine mucosa during the cycle from adolescence through oestrus, pregnancy and lactation to anoestrus comprise alterations in the thickness of the mucosa and its component strata, in the size and abundance of glands and vessels, and in the character of its free surface. In all these points there is considerable variation between the individuals of each group, but the variations between the averages of each group are much more pronounced.

The total thickness of the mucosa (Text-fig 1) is least in immature whales, and rather more than twice as thick in adult anoestrous animals. At or immediately after ovulation there is a striking increase in thickness which becomes nearly three-and-a-half times greater than in the anoestrous adult. This increase is presumably the progestational proliferation brought about by the influence of progesterone produced in the newly formed corpus luteum.



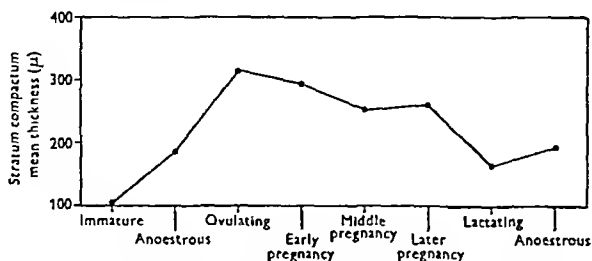
Text fig 1 Mean thickness in mm of the mucosa in the different groups

After ovulation there is a decrease in the thickness of the mucosa which, however, remains thicker than during anoestrus. The thickness is greatest in early pregnancy and thereafter is less. The figures from the present series of specimens show the mucosa to be thinner during the middle part of pregnancy than in early or later pregnancy. This appearance is probably due to the smallness of the number of whales assigned to the middle-pregnancy group, a larger series would probably show a steady gradual decline in thickness of the mucosa from early to later pregnancy, the difference between the figures for the middle-pregnancy group and those for the others in the present series being small. After the birth of the young, but during lactation, the thickness of the mucosa returns to a value not significantly different from that in anoestrous whales.

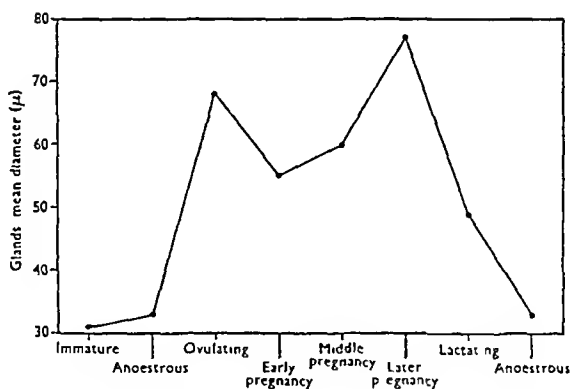
The thickness of the stratum compactum likewise undergoes a cyclic increase and decrease (Text-fig 2). It is greatest during progestational proliferation, but the increased thickness gained during this part of the cycle is not lost so rapidly during pregnancy as the increased thickness of the mucosa as a whole.

There is a steady but slight decline in the figures from ovulation to later pregnancy but it is not great, and is not proportional to the decrease in the thickness of the mucosa as a whole. This retention of the greater part of the increased thickness gained during progestational proliferation is probably to be correlated with the production of the complicated dendritic processes on the trabeculae between the crypts characteristic of later pregnancy.

The diameter of the glands passes through a regular series of changes during the cycle. In the immature animal they are smallest, and in anoestrous adults



Text Fig 2 Mean thickness in microns of the stratum compactum

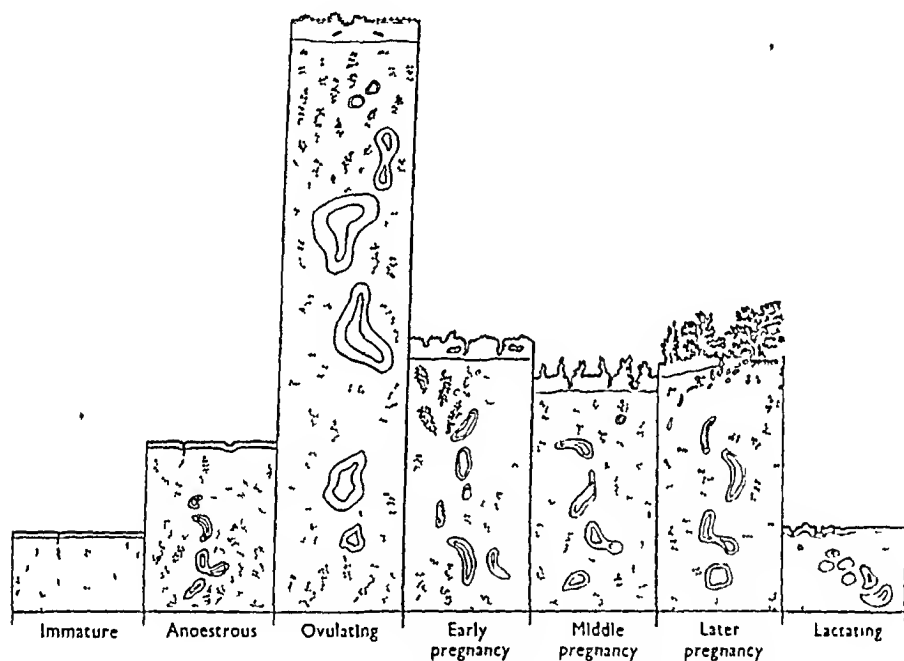


Text-fig 3 Mean diameter in microns of the deeper glands

they are about one third greater in diameter. During progestational proliferation they are greatly increased in size but are slightly reduced again in early pregnancy. During the course of pregnancy they increase again to reach their greatest size during the cycle in later pregnancy when the crypts and dendritic processes too are at their maximum. Two peaks are thus produced in the curve showing these changes graphically (Text-fig 3). After parturition there is a reduction in the diameters of the glands but complete involution to the level found at anoestrus is apparently not quickly attained, for the figures from lactating whales in the present series show gland diameters not greatly less than those of early pregnancy. The two peaks produced in the curve are

caused as much by the appearance of lumina in the glands as by an increase in the size of the lining epithelium. The parts of the glands to which these figures refer are those lying deep in the stratum spongiosum where they are in general rather smaller than in the more superficial parts.

Text-fig 4 shows diagrammatically the quantitative and qualitative changes during the cycle. In immature whales both strata of the mucosa are comparatively thin and the glands have very small or no lumina. The glands, which are confined to the stratum spongiosum, are apparently very much coiled and branched, because although many are seen in transverse section few, indeed, are found to open on the surface. The surface of the mucosa is consequently



Text fig 4 Diagram of the structure of the mucosa in the different groups

smooth with only occasional depressions at the infrequent gland mouths. Vessels are few and small. In the anoestrous adult the mucosa is over twice as thick, but the stratum compactum is only about one-and-a-half times as thick. The glands also are about one-and-a-half times greater in diameter, the increased size being in part due to the greater size of the lumen and in part to the greater size of the epithelial cells. Vessels are larger and more numerous, especially at the base of the stratum spongiosum where many of them have thick walls, being in parous animals the remains of the greatly hypertrophied vascularity of a previous pregnancy. The surface of the mucosa is in general smooth with occasional depressions at the mouths of the glands, very few of which are seen crossing the stratum compactum. As before, the glands must be greatly coiled and branched, for so many transverse sections of them to be present and so few ducts to reach the surface.

Immediately after ovulation, but before the blastocyst has reached the uterus, progesterational proliferation causes a great hypertrophy of the mucosa and its contents. The glands are numerous and closely packed, many having lumina. They are larger in the more superficial layers of the stratum spongiosum, but few ducts cross the stratum compactum to open on the surface. The surface is interrupted by many depressions, representing crypts in the course of formation, which become enlarged so that each depression appears in many sections whereas outlets to the uterine lumen appear in few. The mucosa is highly vascularized with large numbers of vessels both great and small, to such an extent that the vascularity was visible to the naked eye and the uterus was noted in the field as 'very congested'. The surface of the mucosa is invested with a close network of capillaries lying immediately below the epithelial cells. This capillary vascularization of the surface is present throughout pregnancy and gives a very striking and characteristic appearance to sections stained with Mallory's triple stain.

In early pregnancy the mucosa is not as thick and the glands are smaller in diameter. The size of the vessels is also smaller although their number is still great. Tortuous crypts of comparatively large diameter are now more numerous in the stratum compactum, and the surface of the mucosa is considerably incised by the depressions at their mouths. The outline of the deeper parts of the crypts is irregularly serrate in transverse section, as also is that of the outer parts which appear as depressions on the surface of the mucosa. In mid pregnancy this condition has progressed further, so that the surface of the mucosa has taken on a very uneven texture and the tissue between the mouths of the crypts has proliferated to form a large number of branched lobulated processes. The diameter of the glands has again increased and many glands now have easily visible lumina. Vessels are as numerous and as large as in the last stage. In later pregnancy the glands are very large and numerous and have large conspicuous lumina, many of them filled with coagulum. Vessels are now larger and more numerous than in previous stages. The most conspicuous feature, however, is the great degree of complexity reached by the branched dendritic processes of the surface of the stratum compactum. These now greatly resemble foetal villi of the placenta and they form trabeculae with which the villi interdigitated before the chorion was separated from the mucosa. The processes have a slender core of connective tissue in their main trunks, but the branches consist of little beyond capillaries and epithelium. The crypts between the processes are so deep that the stratum compactum has practically ceased to exist as a separate layer and is almost entirely incorporated into the trunks of the processes. In lactating whales the regression of the mucosa has reduced it in thickness practically to that of whales in anoestrus, and the stratum compactum is again visible as a distinct layer, but the surface still retains the remains of the regressing processes so that it presents an uneven outline in sections. The glands, too, have regressed to the size found in anoestrus and are confined to the stratum spongiosum. Vessels are still numerous and large but

many are now very thick-walled and in course of obliteration. A continued decrease in the size of the vessels, and further obliteration of the remnants of the dendritic processes would shortly lead back to the condition typical of anocstrus.

It should be mentioned that although the mucosa becomes highly vascularized during pregnancy and there is produced beneath and among the surface epithelium a close network of capillaries engorged with blood, there is in none of the specimens any indication of spontaneous extravasation of blood either into the stroma of the mucosa or into the lumen of the uterus. This, too, in material which is not in the best histological condition, where the surface epithelium is frequently missing, and signs of histolysis are evident in the deeper tissues.

There appears also to be no spontaneous loss of epithelium, for although it is missing in many of the specimens the abrupt solution of continuity of the epithelium of the gland ducts as they approach the mouth points to the loss being due to post-mortem degenerative changes. Such changes are also almost certainly the cause of the large amounts of cellular debris to be seen in the glands of many specimens. It is noticeable that the parts of the glands in roughly the outer third of the stratum spongiosum are greater in diameter than those of the inner two-thirds and are lined with columnar epithelial cells which are considerably taller. The glands are very closely coiled not only, nor even mainly, spirally but folded up on themselves in a succession of S curves. The complexity of their folding is shown by the great number of transversely cut glands that appear in a single section when compared with the small number of gland mouths that are seen. The same fact indicates the probability that they are extensively branched. It is only during pregnancy, and especially during its later stages, that the ducts appear to be frequent because they are then enlarged and follow tortuous courses in the stratum compactum so that they, too, are cut in many places in a single section.

Where specimens are available from both the pregnant and the sterile cornu of the same whale a tendency is seen for the glands of the pregnant cornu to show greater activity than those of the sterile one. On the other hand, in many specimens the dendritic processes of later pregnancy appear to be even more luxuriantly elaborated in the sterile cornu than in the pregnant one. The reason for this is not clear, but it must be remembered that the chorionic sac of whales extends into the sterile cornu and fills it as well as the pregnant one, so that the villi of the diffuse placenta are inserted into the crypts of both cornua. The activity of the sterile cornu may therefore be quite as important as that of the pregnant one, and as the former is not distended by the presence of the foetus the villi and crypts are not subjected to so great a pressure nor the uterine wall to any considerable stretching. But it is not desirable to be too definite on the point because the specimens are small and represent only a minute fraction of the entire mucosa in the very large uteri, and there is no indication of how representative of the whole the samples may be. The con-

ditions in the single Humpback whale examined agree with these general conclusions as regards the activity of the glands in the pregnant cornu but do not agree in the state of the dendritic processes which are larger in the pregnant than in the sterile cornu. But this specimen corresponds at most to the mid-pregnant group, and in later stages of pregnancy might have resembled the Blue and Fin whales more closely.

DISCUSSION

The absence of the epithelium from the surface of the mucosa is a constant feature of all the whales examined, but it is not, in the opinion of the writer, a true representation of the conditions in life. If the epithelium were absent only during pregnancy one might suppose that a process of denudation was a part of the natural cyclic changes, and that the placenta, though diffuse, was not truly epithelio-chorial. But in view of its constant absence in anoestrous, and even in immature, as well as pregnant whales, one cannot escape the conclusion that the loss is a post-mortem change caused by a maceration of the tissues. Mackintosh & Wheeler (1929) find the same loss in the uterus of the whales that they examined, and state that the 'epithelium is rarely intact over the surface'. All the figures in their plate show the surface without epithelium which is present only in the gland mouths, except in their Fig. 2, where a strip of epithelium lies in a fold of the mucosa from which it has become detached. In view of the fact that in some of the pregnant whales of the present series in which there is extreme proliferation of the dendritic processes of the mucosa the epithelium can be seen in places between the processes, it seems simplest to conclude that in life the epithelium is probably intact over the whole surface of the mucosa. The details of the cycle of changes described above are based on this assumption, and in particular the surface network of capillaries is assumed to be sub- and, to some extent, intra-epithelial from the conditions obtaining in those places where patches of epithelium are present. Mackintosh & Wheeler merely record the fact that the epithelium is absent and do not comment upon it, but their description seems to imply that they consider its loss to be due to post-mortem change. Certainly their figures give this impression. Turner (1871) found in *Orca* and Wislocki (1933) found in *Phocaena* that the epithelium of the mucosa in apposition with the chorion is everywhere present, the cells being cubical (*Phocaena*) or flattened (*Orca*) at the bottoms of the crypts and thin and flattened elsewhere. Turner found that the epithelium was easily lost where the tissues were disturbed and believed that this showed that it was lost spontaneously at a fairly early stage, a suggestion not confirmed by later researches. The conclusion that the epithelium though attenuated is retained is confirmed by the examination of a well-fixed specimen of a Balaenopterid placenta, not part of the present series, which has recently become available for study to the writer and which will be described in a paper on the foetal membranes of Balaenopterid whales now in preparation.

In this connexion it is interesting to note that Haynes & Laurie (1937) found

chorionic region in the porpoise occurs among the present series, in the porpoise this region is described as having the chorionic villi entirely absent or stunted and the mucosa as insignificant in its proportions. In spite of the complexity of crypts and processes in the present series the chorion has been separated from the mucosa in all the specimens without apparent damage to the mucosa or to the chorion, remnants of which are not present as would be expected had damage occurred. The loss of the epithelium from the mucosa of pregnant whales might be attributed to the forcible separation were it intact in the immature and non-pregnant adult whales, but in view of its absence in the latter the loss must be ascribed to the same cause in the former.

Wislocki found that in the porpoise the tufted chorionic villi are applied closely to the mucosa except at their distal ends. The spaces between the distal ends and the mucosa are interpreted by this author as natural spaces into which the glands pour embryotrophic material. The absence of the chorion in the present series makes it impossible to know whether such spaces existed when it was in position, but it is likely that they were present. The glandular stratum spongiosum is very much deeper in all the present specimens from *Balaenopterid* whales than in the porpoise, in which it appears to be everywhere shallow, and in the amnio-chorionic region to be reduced to an inconspicuous remnant. In the *Balaenopterid* whales the glands are obviously in a state of activity which is greatly heightened in the later stages of pregnancy. They then have large lumina, especially in the outer third of the stratum spongiosum, and in the specimens they are filled with coagulum. It is likely, therefore, that the glands produce an embryotrophic material, and that it is secreted into spaces between the chorion and mucosa at the bottom of those crypts into which glands discharge.

Wislocki (1933) points out that the placenta of the porpoise is epithelio-chorial, but that the interlocking of the chorionic villi and the endometrium is much more complex than in the sow. He concludes that at parturition a separation of maternal and foetal tissue without trauma is unlikely, either the endometrium must be torn away to some degree or the chorionic villi must be torn off and retained to a certain extent. This conclusion is not confirmed by the *Balaenopterid* whales in the present series, where in all the specimens from pregnant whales the chorion has been stripped off without apparent damage to the maternal tissues. The condition of the involuting mucosa in the lactating whales supports the view that in these whales damage does not occur. The post-partum involution of both the pregnant and the sterile cornua appears to take place by a resorption of the stroma and a decrease in the congestion without any necrosis or loss of blood or other tissues. In early lactation the processes of the trabeculae can still be recognized, although they are much reduced in size and luxuriance and are no longer dendritic.

SUMMARY

Specimens of the uterine mucosa from the sterile or pregnant cornua, or both from thirty-four antarctic whales have been examined. Data about the state of the ovaries, pregnancy if present, mammary glands and other points were correlated with the conditions found in the mucosa.

A well-marked cycle of changes from the immature state through anoestrus, pregnancy and lactation back to anoestrus was found. The thickness of the mucosa increases greatly in the progestational proliferation immediately following ovulation and thereafter decreases during pregnancy, reaching the thickness characteristic of anoestrus during lactation. The well-marked stratum compactum likewise increases greatly in thickness immediately after ovulation, but does not regress proportionately so quickly as the mucosa considered as a whole. The regression towards the anoestrous state takes place very gradually during pregnancy and rapidly at its termination. The average diameter of the glands in the mucosa increases sharply at ovulation and decreases somewhat during early and middle pregnancy to increase again to a maximum during later pregnancy when the formation of crypts is at a maximum. Regression in gland diameter is rapid during lactation but had not yet reached the involuted state characteristic of anoestrus in any of the lactating whales examined.

Starting with progestational proliferation there is a great increase in congestion in the superficial capillaries of the mucosa. The network of capillaries is so closely developed that in middle and late pregnancy the entire surface of the endometrium is, in effect, covered with a film of blood. In spite of this, no extravasation of blood either into the stroma or lumen of the uterus could be definitely recognized, and was not to be expected in view of the epithelio-chorial nature of the placenta.

The glands are highly contorted and probably also branched, so that in sections of the mucosa vertical to the surface they are cut transversely a large number of times, and the stroma appears to be filled with great numbers of glands. The number of glands, however, is less than at first appears, for the number of gland mouths and of gland ducts crossing the stratum compactum is comparatively small. After ovulation crypts are formed as depressions in the surface of the mucosa. This gives the profile of the mucosa in sections vertical to the surface an irregular serrated outline. As pregnancy advances the stratum compactum is more and more invaded by the crypts. This cutting up of the surface of the mucosa, together with proliferation of the tissue intervening between the crypts, gives the mucosa a spongy structure which appears in sections as a large number of much branched processes. These arise from the trabeculae between the crypts and project into the uterine lumen with a dendritic outline. The dendritic processes reach an extreme degree of complexity during later pregnancy. Gland ducts open at the bottom of some of the crypts. During lactation the processes rapidly regress, but their remains could be

recognized in the uteri of all the lactating whales examined. The regression takes place by a resorption and not by necrosis and surface denudation. The capillary network extends over the surface of the dendritic processes which in their final elaboration become branching fingers interdigitating with the foetal villi.

In all the specimens examined the epithelium bounding the mucosa was generally absent, small patches here and there only being visible. Reasons are given for supposing that this condition is not a natural denudation but is brought about by post-mortem maceration, the processes of degeneration being favoured in dead whales by the insulation of the blubber retaining the body heat after death.

The process of crypt formation is considered, and the conditions now found in the Balaeopteid whales are compared with those already known in the Odontoceti. The distribution of the crypts appears to be more widespread than in the latter, and the crypts are little, if at all, smaller in the amnio-chorionic than in the allanto-chorionic region. The stratum spongiosum is relatively thicker and richer in glands in the Balaeopterids, and the wide lumina holding a coagulum during later pregnancy favour the view that an embryotrophic material is produced and secreted, probably into spaces between chorion and mucosa in the bottoms of the crypts. There is no indication in the present series that there is any damage to maternal or foetal placental tissues at parturition in spite of the complicated interlocking of villi and crypts which must occur. Post-partum involution is rapid and appears to be accomplished without loss of mucosal tissue.

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Uterus musosa

Physiological Group	Wh ref	Surface	Stratum compactum thickness (μ)	Glands		
				Diameter (μ)	Superficial parts if different diameter (μ)	Spacing
Immature	SV	few sulci	200	20-25	—	Wide
	SV		50-60	30-40	—	Wide
	SV	few sulci	80	30-40	—	Wide
Adult in anoestrus	SV		300-400	30-60	—	Very wide
	SV	ridges	100-150	15-30	—	Not wide
	SV	ridges	100-150	40-50	—	Not wide
	SV		200-300	20-30	—	Open
	SV	sulci	150-200	30-55	150-200	Close
			50-60	30-45	—	Open
	SV		200	20-25	30-85	Open
			250-300	15-25	—	Open
	SV					
Ovulating	SV	wrinkled	300-500	60-100	30-50	Close
		wrinkled	150-300	40-70	—	Fairly close
In early pregnancy	SV	large furrows	200-400	40-60	—	Fairly close
	SV	slightly folded	400	20-60	—	Open, superficial third, closer, deeper two thirds
	SV	wrinkled	160-300	50-60	80-140	Rather open
	SV	large folds	280-580	40-80	80-160	Close
	SV		130-140	25-45	—	Fairly close
	SV	folds	250-300	80-100	100-120	Fairly close
In mid pregnancy	SV	to 60 μ	100-170	50-70	60-80	Fairly close
	SV	sulci	200-300	60-80	—	Close
	SV	dentic processes	90-180	40-80	—	Close
	SV	processes 300-400 μ	500-700	40-60	60-80	Wide
In late pregnancy	SV	processes well	230-400	60-80	60-200	Rather wide
	SV	processes to 500 μ	100-300	40-80	150-200	Fairly close
	SV	processes to 1000 μ	200-1000	60-80	120-300	Fairly close
	SV	processes 300-400 μ	500-600	80-100	—	Very close
	SV	processes 600-800 μ	200-400	80-100	100-300	Close
	SV	processes moderate	250-300	40-60	80-120	Fairly close
	SV	processes well	100-200	40-60	—	Fairly close
	SV					

recognized in the uterus of a
takes place by a resorption
capillary network extends
in their final elaboration by
foetal villi

In all the specimens examined
generally absent, small patches
given for supposing that they
brought about by post-mortem
favoured in dead whales by
heat after death

The process of egypt form
in the Balaenopterid whales
Odontoceti The distribution
in the latter, and the egypt
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material is produced and secreted
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in spite of the complicated
Post-partum involution is
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EXPLANATION OF PLATES

All sections of mucosa illustrated are cut vertically to the surface.

PLATE 1

- Fig 1 Mucosa of sexually immature Blue whale SV 47 \times 16.2, showing smooth surface SC stratum compactum SS stratum spongiosum, the glands small and with small lumina I large vessel at base of mucosa
- Fig 2 Mucosa of sexually immature Blue whale SV 280 \times 17.2 showing gland mouths opening on surface D mouth of gland lined by epithelium SC stratum compactum SS stratum spongiosum, some of the glands with lumina
- Fig 3 Mucosa of adult anoestrous Blue whale SV 283 \times 17.2 The section passes through parts of two low ridges SC stratum compactum SS stratum spongiosum, the glands with small lumina I vessel
- Fig 4 Mucosa of ovulating Fin whale SV 102 \times 15.5, showing great thickness and folded surface GL closely packed glands in deeper parts GL loosely packed glands in superficial parts SC stratum compactum with nuclei closely packed near surface and few glands penetrating to it from the stratum spongiosum SS stratum spongiosum I large vessel

PLATE 2

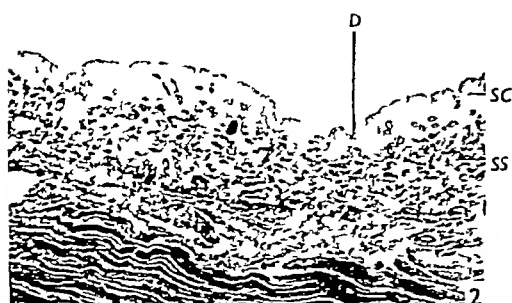
- Fig 5 Superficial part of mucosa of ovulating Fin whale SV 102 \times 17.2 showing comparatively smooth surface SC stratum compactum SS stratum spongiosum with loosely packed glands
- Fig 6 Superficial part of mucosa of ovulating Fin whale SV 102 \times 17.2 showing folded surface with capillaries SC stratum compactum SS stratum spongiosum with loosely packed glands I, vessels
- Fig 7 Stratum compactum of ovulating Fin whale SV 102 \times 17.2 showing surface network of capillaries and loss of epithelium C capillary distended with blood G superficial gland of the stratum spongiosum A, concentration of nuclei below surface of stratum compactum I, vessel supplying capillaries
- Fig 8 Mucosa of pregnant cornu of Fin whale SV 89 \times 17.2 showing smooth surface and large closely packed glands filled with cells in the superficial part of the stratum spongiosum CR crypts in course of formation GS smaller glands in deeper layers GL large glands filled with cells SC stratum compactum SS stratum spongiosum
- Fig 9 Surface of mucosa of pregnant cornu of Fin whale SV 89 \times 17.2 CP small crypts open to the surface CRD oblique crypt cut transversely G large gland filled with cells in superficial part of stratum spongiosum SC stratum compactum

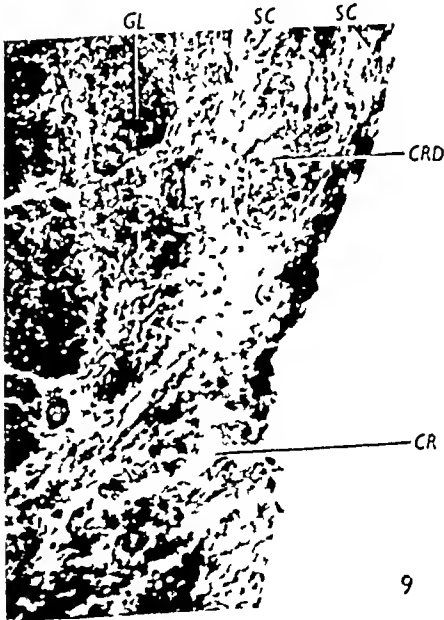
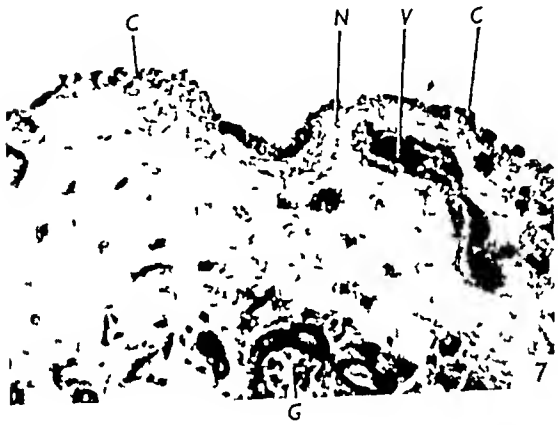
PLATE 3

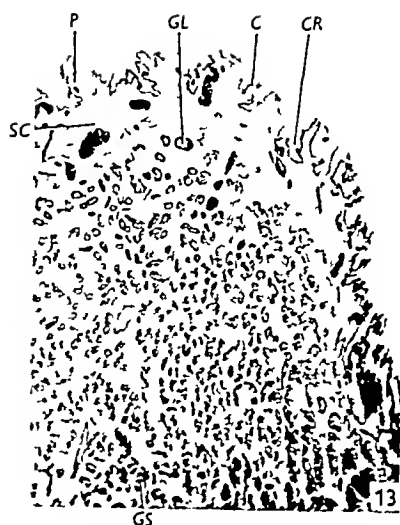
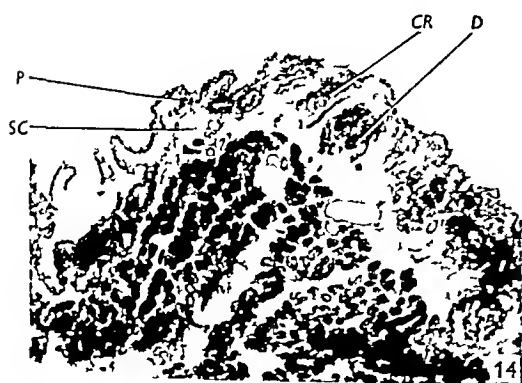
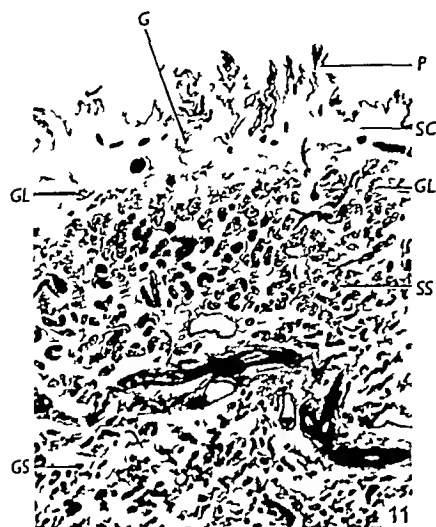
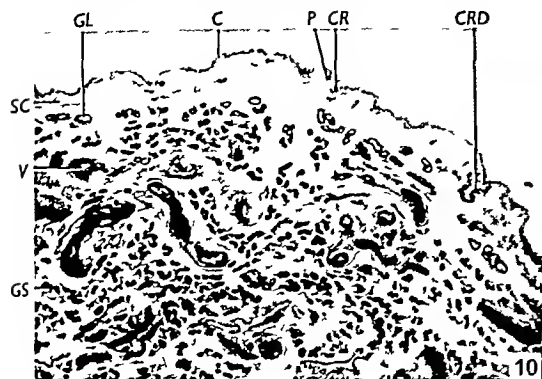
- Fig 10 Mucosa of pregnant cornu of Fin whale SV 472 \times 17.2 C capillaries CP crypt CPD oblique crypt cut transversely GL larger superficial gland with lumen GS smaller deep gland with no lumen P, lobulated process between incipient crypts SC stratum compactum I, vessels
- Fig 11 Mucosa of pregnant cornu of Fin whale SV 164 \times 17.2 G gland opening into the bottom of a crypt GL larger superficial glands GS smaller deep glands P, lobulated process between crypts SC stratum compactum SS stratum spongiosum
- Fig 12 Mucosa of pregnant cornu of Fin whale SV 164 \times 136.3 showing surface of branched process with capillary network C capillary distended with blood I larger vessel
- Fig 13 Mucosa of sterile cornu of Blue whale SV 247, \times 17.2 C capillary network CP crypt separated from each other by branched processes GL larger superficial gland GS smaller deep glands P, lobulated process SC stratum compactum
- Fig 14 Mucosa of pregnant cornu of Blue whale SV 44 \times 17.2 showing the large gland of the superficial layers of the stratum spongiosum, and lobulated process between the crypts. The processes are in a stage of formation intermediate between those shown in figs 10 and 11 CP crypts in course of formation D tortuous gland duct entering the stratum compactum to open into a crypt P, lobulated process SC stratum compactum

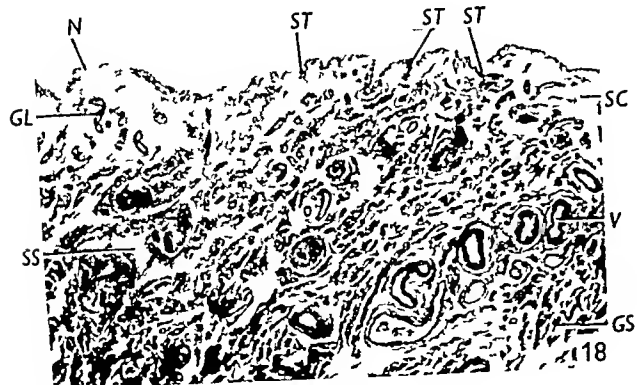
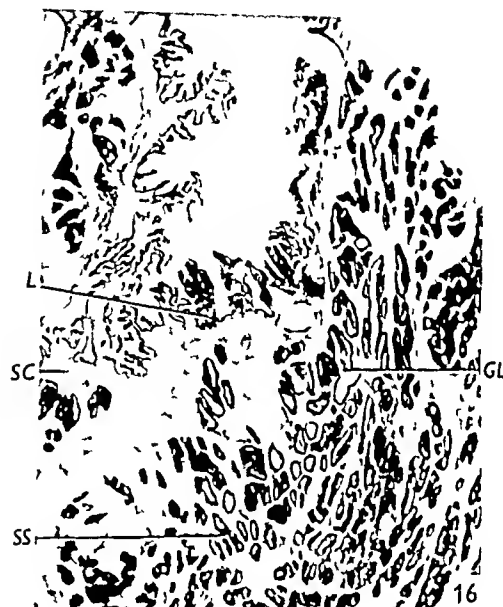
PLATE 4

- Fig 15 Mucosa of sterile cornu of Blue whale SV46, $\times 17.2$ *GL*, large superficial glands *GS*, smaller deep glands *P*, branched dendritic process *SC*, stratum compactum *SS*, stratum spongiosum *V*, vessel
- Fig 16 Mucosa of pregnant cornu of Fin whale SV40, $\times 17.2$ *GL*, large superficial glands with large lumina *L*, bases of some processes infiltrated with leucocytes *SC*, stratum compactum *SS*, stratum spongiosum
- Fig 17 Mucosa of sterile cornu of Blue whale SV48, $\times 17.2$, showing extreme proliferation of the dendritic processes *GL*, large superficial glands with very large lumina *SC*, stratum compactum nearly completely included in the dendritic processes by the extension of the crypts to its base *V*, vessel
- Fig 18 Mucosa from the cornu which had probably been sterile in freely lactating Blue whale SV245, $\times 17.2$ *GL*, glands of superficial parts now much reduced in size *GS*, smaller glands of deeper layers greatly reduced *N*, nuclei concentrated at surface of stratum compactum *SC*, stratum compactum with no surface capillary network *SS*, stratum spongiosum *ST*, strand representing partly obliterated crypt *V*, vessels with thick walls some in course of obliteration
- Fig 19 Mucosa from presumed formerly sterile cornu in slightly lactating Blue whale SV127, $\times 17.2$ *G*, glands now few and small *P*, remnants of dendritic processes between reduced crypts *SC*, stratum compactum *V*, thick-walled vessels in course of obliteration









THE LOCAL ACTION OF THE PARATHYROID AND OTHER TISSUES ON BONE IN INTRACEREBRAL GRAFTS

By N A BARNICOT, *University College, London*

INTRODUCTION

The conception that the parathyroid hormone acts directly upon the skeleton by stimulating osteoclastic resorption has not passed unchallenged. As a result of studies such as those of Albright & Ellsworth (1929) and Harrison & Harrison (1941) on changes in the rate of excretion of calcium and phosphate soon after parathyroid extract administration, and of the effect of these extracts on the serum calcium and phosphate in nephrectomized animals, by Neufeld & Collip (1942), some workers have advanced the view that the hormone has a primary action on the phosphate threshold of the kidney, and that bone resorption is a secondary effect mediated by changes in the calcium-phosphate equilibrium in the plasma. Although later work by Selve (1942), Ingalls, Donaldson & Albright (1943), and others, has shown that there is histological evidence of osteoclastic resorption even in nephrectomized animals treated with parathyroid extract, the experiments described here provide more direct additional evidence of the local action of the hormone on the skeleton. Nevertheless, they do not exclude the possibility that the hormone affects the kidney also and this action may still be the most important in the intact normal animal.

It seemed possible that if parathyroid glands were brought into close contact with bony tissue the local concentration of hormone might be sufficiently high to produce resorption of the immediately adjacent bone. Small pieces of the parietal bone of 10-day-old mice were used, and glands from the same animal were attached to their endocranial surface with plasma clot. The combined graft was then inserted into the cerebral hemisphere or meninges of a litter-mate. The advantage of this procedure is that the soft-tissue graft is maintained in very close contact with the osteogenic layer of a fairly uniformly thin and solid piece of bone. Any change in the degree of deposition or resorption by this layer in the vicinity of the gland should then be clearly evident by comparison with the regions on either side. It would be less satisfactory to graft the gland to some exposed bone surface in the intact animal, because intervening connective tissue would prevent such close contact, moreover, the very small soft-tissue grafts would be difficult to locate at the end of the experiment, and would be more liable to mechanical disturbance than they are when living protected within the cranial cavity. If the parathyroid or some other tissue is grafted in this way, and a reaction of the adjacent bone is observed, this cannot be attributed to an indirect mechanism involving a change in the activity of some distant organ. It is possible that the graft in addition

exerts some systemic effect which may play a part in conditioning the response of the grafted bone, but some purely local action must also be operative. It does not follow, of course, from the evidence provided by this type of experiment alone, that the grafted soft tissue exerts a local effect on the bone by means of a specific secretion, or that its action is one which is significant in the normal physiological regulation of the skeleton. It will be shown that particularly as regards bone deposition, the interpretation of the results may be complicated by non-specific degenerative changes.

MATERIAL AND METHODS

Tissues for grafting were removed from 8- to 10-day-old mice and implanted in the cerebral hemisphere of the litter-mates. In the course of the work, animals from several strains have been used, these include normal and mutant animals from an inbred and also a recently outcrossed grey-lethal stock from an inbred C57 black stock, and also from an albino stock of doubtful genetic purity. In addition, a number of rats from a retinitis pigmentosa strain were employed. The number of experiments is too small to allow any conclusions regarding the effect of genetic homogeneity on the success of the grafts.

Aseptic precautions were observed throughout. For parathyroid grafts the larynx and attached thyroids were removed to 0.9% saline. The thyroid lobes were detached and examined under the binocular dissecting-microscope, using reflected light and a black background. The parathyroids can usually be detected as somewhat whiter, more compact bodies, lying partially embedded in the lateral aspect of the lateral lobes of the thyroid rather nearer to the superior pole. In pigmented strains they are sometimes identifiable by a large branching pigment cell. They can be removed with needles, and much of the adherent thyroid dissected away. To prepare the bone grafts, the skull roof of the same animal was removed with scissors into 0.9% saline, and pieces about 3-4 mm square were cut from the parietal. These were laid with their concave or endocranial surfaces uppermost in small drops of saline, the parathyroid was then transferred to the centre of a bone fragment, the excess saline drained away and fowl or mouse plasma rapidly placed on the graft. Clotting generally occurred without addition of tissue extracts. In later experiments dried human fibrinogen and thrombin, with the addition of penicillin, were used with excellent results. A litter-mate, from which the scalp hair had been clipped and the scalp sterilized with tincture of iodine, was anaesthetized with ether. The skull roof was exposed by a median incision, the skin retracted, and a small rectangular hole cut with scissors in the posterior end of the left parietal. A piece of the protruding brain tissue was excised with the scalpel and the graft, held at its edge in fine forceps, was inserted through the hole and pushed forward below the host parietal. The skin wound was sealed with collodion and the host animal was returned to the mother when sufficiently recovered to move actively. The hosts were killed 10-14 days later, and the cerebral hemispheres of the graft side, together with the skull roof were excised with a razor.

blade and fixed for 24 hr in Zenker+3% glacial acetic acid, which both fixed and decalcified the material. The specimens were infiltrated and embedded in celloidin, and the celloidin blocks were then infiltrated with paraffin wax, and cut serially through the graft region at 8-10 μ . Some sections were stained with Masson's triple stain, but for the majority haematoxylin and Orange G-Erythrosin was used.

(1) *Normal parathyroid and bone grafts into normal hosts*

The following account is based on twelve mouse and four rat grafts in which the grafted tissues were located in sections and found to have survived. In addition, two specimens were prepared as alizarin transparencies and subsequently macerated and examined dry in order to obtain a better overall view of the bone structure of the grafts (Pl 1, figs 1, 2).

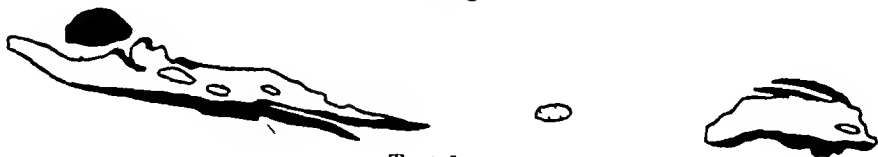
In all of the eighteen specimens there is evidence of osteoclastic resorption of the bone graft in the neighbourhood of the parathyroid, although the magnitude of the effect varies considerably. In twelve specimens the graft bone is actually perforated. The position of the grafts was by no means constant, some lay largely embedded in brain tissue, sometimes close to the lateral ventricle others rested in the meninges immediately below the host calvarium. The posterior end of the bone graft often lay in the scar tissue which filled the hole in the host parietal.

It is convenient to describe the results in several groups, each comprising specimens similar in their histological appearance. The first group, illustrated by Text-fig 1 and Pl 1, figs 1, 3 and 4, includes five mouse and one rat graft. In Pl 1, fig 3, the graft lies in the meninges and in Pl 1, fig 4 is embedded more deeply in brain tissue. Characteristically, the parathyroid is in good condition, without evidence of degeneration. The gland is judged to be at least as large as that of an unoperated animal of this age and consists of cords of basophilic cells separated by capillaries, a varying amount of residual thyroid tissue is found adhering to it. In this batch of material the gland lies surrounded by a loose connective tissue in which the ratio of fibroblasts to fibres is high and macrophages or other inflammatory cells are infrequent. The fibroblasts and the fine collagen fibres which are best seen in Masson-stained sections have a predominantly radial arrangement around the gland.

The region of the bone graft adjacent to the parathyroid is not perforated, or only incompletely so, but consists of a thin layer of reticulate bone, which stands in contrast to the thicker, more compact and often more strongly staining bone on either side (Text-fig 1, Pl 1, figs 3, 4 and 6). This zone of reticulate bone tends to form a semicircle around the gland, the radius of curvature of which is often less than that of the original bone implant. As seen in the macerated specimen (Pl 1, fig 1) it therefore appears as a circumscribed rounded projection on the convex aspect of the graft bone. Its surface nearest to the parathyroid, that is the original endocranial surface is lined with numerous osteoclasts. In some specimens (Pl 1, fig 6) one finds some of these



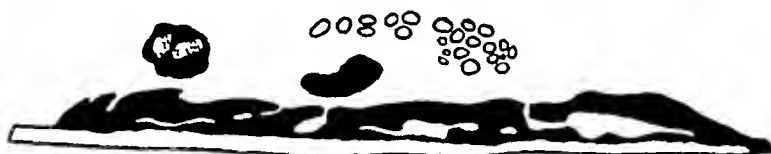
Text fig 1



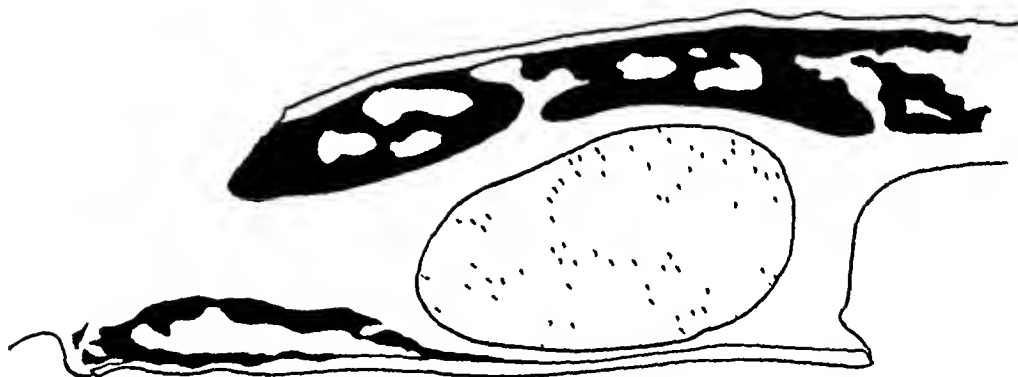
Text fig 2



Text fig 3



Text fig 4



Text fig 5

Text figs 1-4 Projection drawings. Bone deposited on the graft after implantation is marked in black, the original graft bone is white. All the specimens are from grafts of normal tissues into normal hosts. Text fig 1 Parathyroid graft. Complete destruction of the original graft bone opposite the gland, and formation of new bone. Some thyroid vesicles adherent to parathyroid. At the sides, the thickest new bone deposition is on the concave aspect of the graft. Magn $\times 94$. Text fig 2 Parathyroid graft using rat material. The bone graft is extensively perforated. Magn $\times 54$. Text fig 3 Pituitary graft. Anterior lobe tissue. There is fairly uniform deposition of new bone over the concave aspect of the bone graft. Magn $\times 94$. Text fig 4 Thyroid graft. There is considerable, uniform deposition of spongy new bone over the bone graft surface. Two detached bone nodules are probably ossified necrotic tissue, and the left hand one contains granular material. Magn $\times 94$. Text fig 5 Adrenal graft. The area of the bone graft against which the necrotic adrenal lies shows no deposit of new bone in contrast to the thickened area on one side. Magn $\times 94$.

osteoclasts orientated at right angles to the bone surface in conformity with the general direction of fibroblasts, and individual cells are sometimes seen, which might well be transitional between mesenchymal cells and osteoclasts. On the opposite or convex side of the reticulate bone is a layer of large, quadrangular, basophilic osteoblasts. It appears that the bone graft is being actively eroded on its concave surface while new bone is being deposited on its convex side. Whether or not actual perforation occurs will evidently depend on the balance between these two processes.

The contrast between the bone of the eroded zone and that on either side is rather sharp. The contrast in thickness is, however, exaggerated by the occurrence of new bone deposition, particularly on the concave surface of the graft in the peripheral regions around the parathyroid. It is usually possible to distinguish the original bone graft by the cement-lines which demarcate it from this secondary deposit. The original graft appears as a uniform, solid sheet, the osteocytes of which are commonly dead. In some cases its surface appears to have been partly eroded prior to the deposition of new bone on it. It is important to rely more on a detailed study of the histology to distinguish new from original bone, than on measurements of thickness, because the grafts may be cut somewhat obliquely if they be embedded in brain tissue, and because the parietal fragments were not always from exactly the same region and the structure and thickness of this bone is not uniform throughout its extent, as shown by Barnicot (1947). Many grafts also show new bone deposition along the entire convex surface, but this bone is thin and compact, whereas that on the concave surface is often spongy and much thicker (Text-fig. 1).

The second group of six specimens, three of them from rats, is illustrated in Text-fig. 2 and Pl. 1, figs. 2, 5, and shows complete perforation of the bone graft opposite the parathyroid. The hole is often extensive and exceeds the diameter of the gland. It is closed by a fibrous membrane in which osteoclasts can sometimes be found even in the absence of bone spicules. The edges of the hole are usually quite sharp, and a few osteoclasts occur in this position. The parathyroid is free from degenerative change, but is often more compact in structure, and is surrounded by a more densely fibrous connective tissue than those in the first group described above. In two cases the gland is pressed between the fibrous membrane and overlying brain tissue. This group of grafts gives the impression that the activity of the resorptive process has reached a climax and died down, with accompanying fibrosis and perhaps diminished vascularity.

The remaining grafts present certain peculiarities which may be briefly described.

Two of them show parathyroids involved in a necrotic process (Pl. 1, fig. 7) and the living parathyroid tissue remains as only a few strands lying in a rather dense connective tissue. Nevertheless, there is perforation of the adjacent bone and large osteoclasts on its concave surface adjacent to the gland. In one specimen, overlying necrotic brain tissue shows giant cells on its deep surface,

and it is interesting to note the similarity of these to the osteoclasts on the nearby surface of the bone graft. It is impossible to say how far the observed bone resorption was accomplished prior to the partial necrosis of the gland but considering the relatively small amount of new bone deposition either in the eroded zone or elsewhere, it is likely that the grafts never passed through an active phase comparable to group I specimens. It is, therefore, worth noting what small fragments of parathyroid tissue can produce resorption, even when the condition of the graft is poor and necrosis widespread.

There are three specimens in which, although the parathyroids are large and free from necrotic change, the adjacent bone shows only comparatively slight resorption and only a few osteoclasts. In one of these the parathyroid lies in rather dense connective tissue below the hole in the host skull, while in another it is unusually closely surrounded by brain tissue. In the third the well-preserved parathyroid is embedded in a large fragment of thyroid. The intention had been to graft thyroid only, and the parathyroid was inadvertently included. Although the parathyroid lies quite close to the bone surface, the original graft bone is probably scarcely resorbed at all, and the osteoclasts, although localized in this position, are few in number.

Three grafts, one of which is illustrated in Pl. 1, fig. 8, were examined 2 days after implantation. The graft bone is thin, and its surfaces only slightly eroded. It is much buckled and distorted, and lies among blood clot and fragments of injured brain tissue. On its concave surface the layer of osteoblasts is seen to be more or less disorganized, and mesenchymal cells are growing out into the fibrin clot as if in tissue culture. No osteoclasts are to be found, and many of the osteocytes are dead. In two cases the parathyroid was located and showed evidence of degenerative change. Some of the nuclei were pyknotic and some of the cells swollen and granular. Capillaries were observed between the epithelial cells, but the surrounding tissue was apparently not vascularized. Both glands lay close to the bone surface but there was no local osteoclastic resorption.

One may now consider some general points of interpretation of this material. Apparently the resorptive and osteogenic activities seen in older grafts have not started at 2 days, and it is probable that they do not commence until vascularization is established. It is possible, however, that the glands, which at 2 days after the operation would be only 10–12 days old, are not yet capable of secretion. Foster (1943) has shown that, in the mouse, a series of cytological changes leading to the establishment of the adult condition is initiated at 10–15 days after birth. It would be interesting to check the activity of the glands as inferred from cytological structure, by means of the functional test provided by the present technique. The semi-regular arrangement of the zone of erosion and osteogenesis around the gland suggests that resorption starts and is most active in the region immediately adjacent to the gland. It may be noted that the numerous osteoclasts in this site are certainly newly differentiated, since in a 10-day mouse the central region of the parietal is substantially free

from them, as shown by Barnicot (1947), and in the 2-day grafts any osteoclasts which may have been present on the implants had evidently disappeared. The intense osteoclastic activity shown in some of the grafts is accompanied by equally intense bone formation only a few microns away. One is prompted to ask whether the secretions of the parathyroid are responsible for both processes, the dynamic equilibrium between them being speeded up. It is well known that in animal experiments the injection of large doses of parathyroid extract is followed by osteoclasia and also by the conversion of the osteoblasts to spindle cells which are presumably inactive. However, it has been shown that the successive injections of small doses may lead to an actual increase in the amount of bone trabeculae at the metaphyses. It may be, on the other hand, that in the present material the active bone formation in the zone of resorption is a secondary effect of mechanical stimuli due to the local thinning of the graft. In this case the osteoblasts must be thought of as remaining capable of bone formation, because the mechanical stimulus outweighs the inhibitory action of the hormone which must presumably reach them, particularly when the reticulate bone layer is perforated at intervals. It seems probable that the bone grafts are subject to an intracranial pressure tending, when they lie in the meninges, to reverse their curvature and flatten them against the host skull. In a few cases the edge of the graft where it lies against the host parietal is surrounded by a local area of bone resorption which may be taken as indicating such pressure. The layer of reticulate bone, however, often indents the brain surface, and must advance against this pressure. The loose character of the connective tissue, and its radial orientation around the parathyroid, argue against the eroded zone being subject to pressure from this direction. The question to what extent the mechanical pressure exerted on the bone by the soft-tissue graft may be instrumental in producing local resorption, is taken up again in discussing experiments with other tissues, particularly those using cartilage.

The deposition of new bone on the concave surface of the grafts in the regions peripheral to the zone of erosion might also be attributable to the action of the parathyroid. This is, however, a feature commonly found in grafts of other tissues also. The amount and character of this new bone seems to be conditioned by various factors which affect all grafts to a greater or less extent. A deposition of spongy new bone is often most marked where the graft lies in scar tissue in the hole in the host skull, while more anteriorly where it may be embedded in the hemisphere and closely surrounded by brain tissue, the new bone is often thinner and in the form of a continuous layer. Probably inflammatory changes and the presence of a certain amount of organizing necrotic tissue, provided they are not so intense as to lead to the death of the osteogenic cells of the graft, favour the proliferation of this new bone, which becomes particularly thick and acquires a complex spongy structure if the adjacent tissue is loose and does not impede its advance. As described above, the osteogenic layer of the grafts, as seen at 2 days, is somewhat disorganized

and osteogenic cells have probably become widely dispersed in the plasma clot by the time vascularization is established. The frequent ossification of necrotic soft-tissue grafts, which is mentioned below, and also the frequently spongy character of the new bone deposit, on the concave surface, may perhaps be partly accounted for by this fact. The close apposition of the brain against the convex aspect of grafts in the meninges may perhaps explain the thinner, more compact structure of the new bone on this surface.

It is probable that the effect of the parathyroid on the bone graft is due to hormone carried to it in blood vessels, and various other features of the material may be attributable to the arrangement of vascular connections. The semicircular form of the zone of resorption round the gland might suggest a diffusion of the active principle, particularly in two cases (Pl. 1, fig. 3) in which the host skull also was eroded, thus completing a circle of resorption. However, in both these specimens an 'osteitis fibrosa' tissue rich in spindle cells and osteoclasts, can be traced into the diploe of the host parietal which, as is usually the case, is much thickened on the side where the implant was made. This seems to be explicable only if the hormone is conveyed by the blood vessels, and the presence of spindle cells in this position, where mechanical factors are probably less important, is in significant contrast to the active osteoblasts in the zone of resorption of the graft bone. The reverse situation is illustrated in Pl. 1, fig. 5 where, although the gland lies very close to the host skull, no resorption is seen at this site. It will be noted that an artificial space separates the meninges from the skull roof, and in all probability it is the absence of vascular connections which accounts for the lack of resorption here, while the bone graft is being actively eroded. It has already been suggested that the absence of resorption in 2-day grafts and the diminished activity in some perforated grafts are due to the condition of the blood supply, which in the former is not yet established, and in the latter has become diminished. Capillaries can sometimes be traced from the parathyroid graft into the surrounding connective tissue; the zone of active resorption does not appear to be particularly rich in blood vessels, but injected specimens would be required before much could be said with certainty about the arrangement and richness of vessels in the grafts. Since the posterior end of the bone graft is usually in the hole in the host skull it is not unlikely that blood vessels grow in from here, and this may explain the fact that connections are not always established with the overlying host skull. The cases in which resorption is slight in spite of the survival of the parathyroids may also perhaps find an explanation in the vagaries of vascular supply.

(2) *Parathyroid-bone grafts with grey-lethal material*

The skeleton of the mutant grey-lethal mouse, described by Grunberg (1936) is remarkable for the excess of trabeculae in the marrow cavities and various abnormalities in the shape of the bones, due to very defective secondary resorption during development. Barnicot (1941, 1945) has shown that much

of this excess bone is resorbed if grey-lethal bones are grafted subcutaneously to normal hosts, or if grey-lethal mice are given doses of parathyroid extract in excess of those required to produce resorption in normal animals

The following combinations with the present technique were set up with a view to obtaining more information about the activity of the grey-lethal parathyroid which, as far as is known, is structurally normal

	Implant		Host
	Parathyroid	Bone	
(i)	Grey lethal	Grey lethal	Normal
(ii)	Grey lethal	Grey lethal	Grey lethal
(iii)	Normal	Normal	Grey lethal

The whole batch of experiments, particularly those in which grey-lethals were used as hosts, yielded few successful grafts, but, although the material is scanty, certain conclusions can be drawn from the results, which are dealt with here since further experiments are unlikely to be undertaken by the writer

(i) Three specimens are available in which living parathyroid tissue was located. All three show definite evidence of osteoclastic resorption in the neighbourhood of the gland. In one (Pl 2, fig 9) resorption is accompanied by osteogenesis on the convex aspect and the picture is altogether comparable to group I grafts of normal tissues. It will be noted that there is little or no evidence of erosion of the overlying skull and thus, as already suggested, may be due to lack of vascular connexions. In the second specimen, illustrated in Pl 2, fig 10, the graft is embedded in the brain. The parathyroid is smaller and more compact and lies on a fibrous membrane which bridges a sharp edged hole in the bone graft, at the edges of which are a few osteoclasts. In the third case, the parathyroid is embedded in brain tissue and lies some distance from the bone graft, which, none the less, shows some resorption and many osteoclasts on the region opposite the gland, although the bone is not actually perforated. This specimen, and the previous one, are consistent with the view that it is by vascular connexions rather than by direct diffusion that the hormone reaches the bone.

From these specimens it can be concluded that the grey-lethal parathyroid is capable of producing its secretion, at least in the environment provided by a normal host. It is not surprising to find that the grey-lethal bone responds, since it is already known that the grey-lethal bones exhibit resorption when grafted alone to a normal host. The sharp localization of the resorption in these experiments shows that it is due to the gland. The results are also in harmony with the previous work in which resorption in the bones of grey-lethal mice was shown to occur after repeated massive doses of parathormone. It is evident that in the intact grey-lethal mouse the bones do not respond to the animal's own parathyroid, this may be because under these conditions the activity of the gland is subnormal or because the bones are relatively insensitive, or both. On the other hand, it may be that implantation of bone and parathyroid in a normal host results in no alteration in the activity of either tissue,

Thyroid grafts

It was felt desirable to investigate grafts of thyroid alone, partly because some of this tissue was adherent to the parathyroid grafts, but also because the osteoporosis which sometimes occurs in hyperthyroid cases perhaps indicates a stimulation of bone resorption. Robertson (1942), however, regards the increased urinary calcium excretion in hyperthyroidism as due to a change in the renal threshold, and Albright (1947) suggests that the osteoporosis is due to a negative nitrogen balance which limits bone-matrix synthesis.

The account is based on twelve specimens, in which pieces of thyroid about 1.0 mm square, cut from the gland with scissors, were used for grafting. There was no evidence of local osteoclastic resorption, but as in the pituitary grafts, the deposition of new bone appeared to have been impeded by the graft, particularly if the latter retained a compact structure. Evidence of considerable degeneration was found in all grafts of thyroid tissue, and even in the best specimens (Pl. 2, fig. 15) the surviving vesicles were abnormally distended with colloid, and it may be questioned whether they were secreting hormone into the blood stream. In three specimens (Text-fig. 4) there were free nodules of bone in the thyroid which were apparently due to the ossification of necrotic tissue.

Adrenal grafts

The use of whole adrenals, or even of the bisected gland, for grafts of this kind is technically difficult because of their large size. Even in successful cases in which the glands retained their position on the graft bone, it was found that very extensive degeneration had occurred, and, following the procedure of Williams (1947), small fragments of capsule were used for the last three of the ten specimens on which this account is based. The specimen illustrated in Text-fig. 5 shows very well the mechanical effect of a large, compact, but almost entirely degenerate mass of tissue, in restricting the formation of new bone both on the adjacent area of the bone graft, and on the overlying host skull. The other specimens of whole or bisected glands showed similar effects. In two of the capsule grafts (Pl. 2, fig. 16) a small mass of regenerated adrenal, intermediate in structure between glomerulosa and fasciculata tissue, was found. There is no good evidence that this tissue produced any specific effect on bone deposition or resorption in its vicinity, the very massive proliferation of spongy new bone in all three of these specimens, leading to synostosis with the host skull, is probably attributable, as its detailed structure suggests, to ossification of fragments of necrotic adrenal tissue, which had been introduced in a fragmented condition.

Cartilage grafts

One graft was performed using rib-cartilage from the zone of endochondral ossification, and two others using blocks of cartilage from the non-ossifying zone. The former underwent conversion to a nodule of spongy bone. The

adjacent bone graft showed no local resorption but the nodule was not closely pressed against it. One piece of non-ossifying cartilage became synostosed to the new bone which surrounded it, the other (Pl 2, fig 17) lay free but closely pressed against the bone graft. It is clearly seen that the deposition of new bone on the bone-graft surface is minimal where the cartilage lies in close contact with it, while to one side a considerable thickness of new bone has been formed. If mechanical pressure were an important factor in producing local resorption in grafts of this kind, one might expect to observe it in cartilage grafts because of the harder consistency of the tissue. The fact that osteoclastic resorption was not seen renders it unlikely that mechanical pressure was important in producing the resorption described in parathyroid grafts.

SUMMARY

1 Parathyroid glands, dissected from 10-day-old mice, have been attached to pieces of parietal bone from the same animal and grafted to the cerebral hemisphere of a litter-mate.

2 After 10-14 days it was found that very active bone resorption accompanied by osteoclasts was occurring on the surface of the bone graft opposite the parathyroid. This resorption was often accompanied by new bone deposition on the opposite surface of the bone graft, while in other specimens actual perforation occurred. It is concluded that the parathyroid hormone is capable of stimulating osteoclastic resorption by a direct action.

3 Similar graft combinations utilizing grey-lethal mutants as donors and hosts are described. It is shown definitely that the grey-lethal parathyroid can produce local resorption of grey-lethal bone when these are grafted to a normal host.

4 Grafts utilizing pituitary, thyroid, adrenal and cartilage tissue are described. In no case was there evidence of local resorption of bone. Most of the observed effects are attributed either to the mechanical restriction imposed by the soft-tissue graft on the deposition of new bone which always tends to occur on the surface of the bone graft, or to the invasion and ossification of necrotic tissue.

I wish to thank Dr Gruneberg for providing me with certain of my mouse stocks, Mr Warwick James for the loan of a sliding-microtome, and Prof J Z Young for the facilities provided by the Department of Anatomy during the completion of this work, while I was attached to the Department of Anthropology. I am also much indebted to Miss O Occomore and Mr A Kozlowski for help with the technical work, to Mr F J Pittock for the photographs, and to Miss K E Attwood for typing the manuscript. The work was partly accomplished during the tenure of a grant from the Dixon Fund of the University of London.

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EXPLANATION OF PLATES

PLATE 1

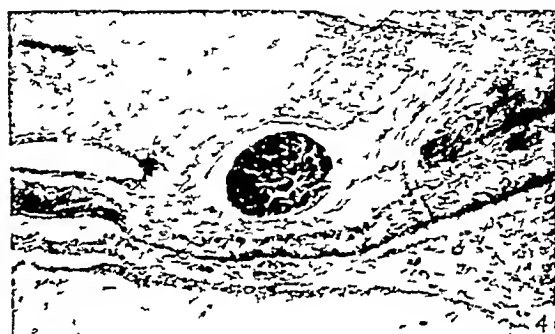
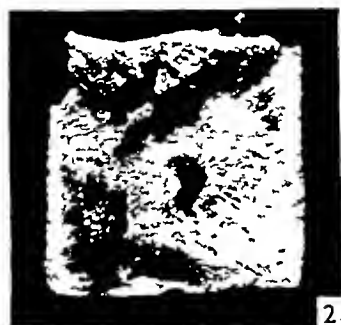
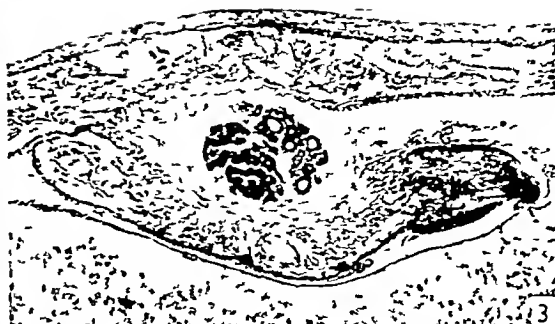
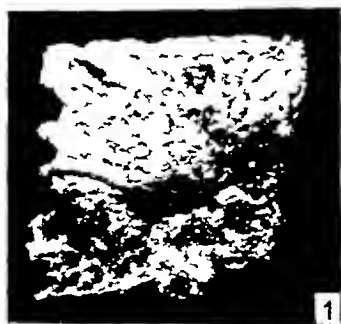
- Fig 1 Macerated bone graft 14 days after grafting, with attached normal parathyroid, to a normal host. Convex, ectocranial aspect, showing the bulging and circumscribed zone of new bone formed opposite the parathyroid graft. Magn $\times 20$
- Fig 2 A similar graft to that in Fig 1, seen from the concave, endocranial aspect. Perforation has occurred. Note the thickening by new bone deposition round the periphery of the graft, particularly on two sides. The upper side lay within the scar tissue of the hole in the host skull. Magn $\times 20$
- Fig 3 Graft of normal bone and parathyroid into a normal host. There is marked osteoclastic resorption opposite the gland, accompanied by new bone formation on the convex surface. Resorption is evident on the adjacent, thickened, host skull, and at the left hand side the diploic space is enlarged and contains 'osteitis fibrosa' tissue. Some thyroid vesicles are adherent to the parathyroid. Note the indentation of the cerebral surface by the graft. Osteoclasts have been emphasized by inking. Haematoxylin Orange G Erythrosin. Magn $\times 85$

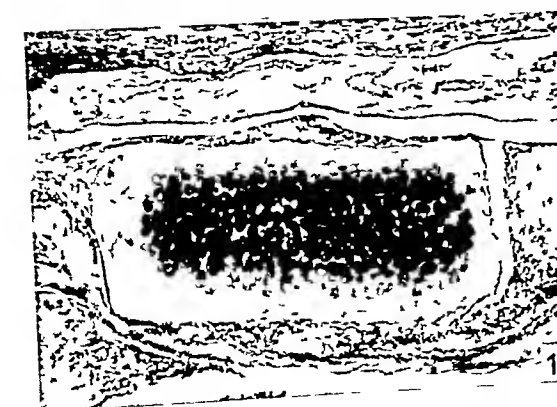
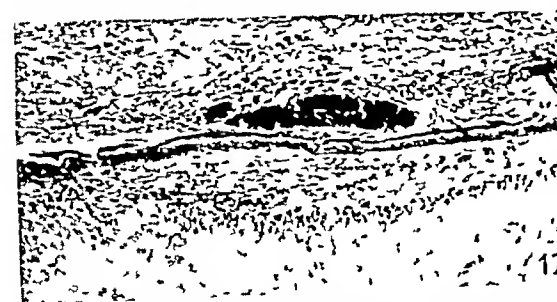
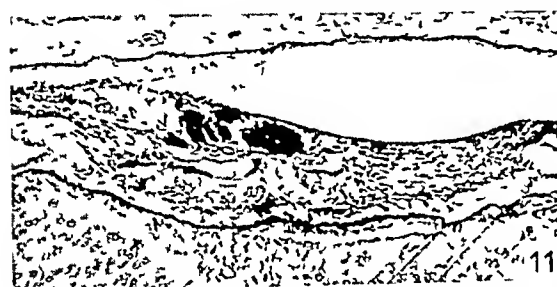
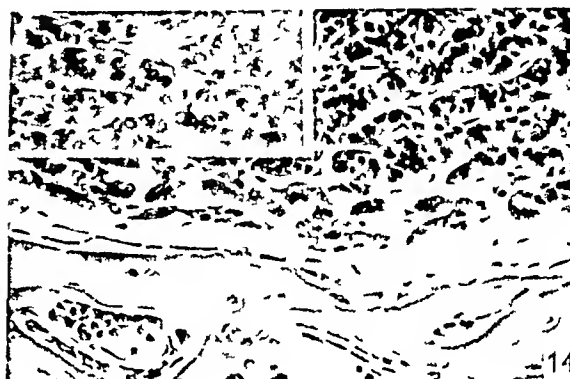
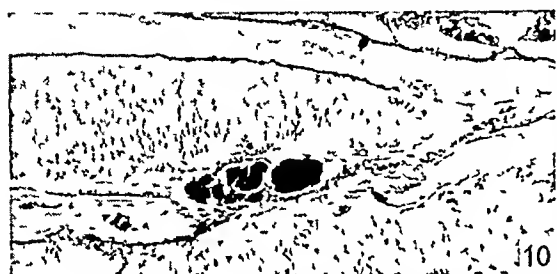
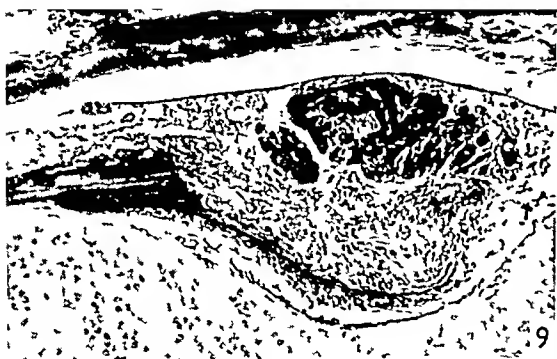
- Fig 4 Graft of normal rat bone and parathyroid to a normal litter mate Opposite the gland a layer of new, reticulate bone, which is lined with osteoclasts (marked with ink) on its concave aspect, has entirely replaced the original graft bone The original bone, contrasting in staining and structure, is seen on either side The graft lies embedded in brain tissue Haematoxylin Orange G Erythrosin Magn $\times 85$
- Fig 5 Graft of normal bone and parathyroid into a normal host The bone graft is perforated opposite the parathyroid The hole is bridged by connective tissue which is rather more fibrous than that surrounding the gland in Fig 3 The host skull shows no, or very slight resorption Haematoxylin Orange G Erythrosin Magn $\times 60$
- Fig 6 High power view of the central region of the zone of resorption shown in the specimen Fig 3 At the right hand side note the osteoclast orientated at right angles to the new bone layer, in conformity with adjacent fibroblasts Haematoxylin Orange G Erythrosin Magn $\times 380$
- Fig 7 Graft of normal bone and parathyroid to a normal host The parathyroid has been much reduced by necrosis so that only the small strip, seen in the upper part of the field, remains There is, however, perforation of the adjacent bone graft and at the edge of this perforation, several large osteoclasts are seen on the bone Haematoxylin Orange G Erythrosin Magn $\times 380$
- Fig 8 A normal parathyroid grafted with normal bone into a normal host and sectioned 2 days after implantation The nuclei are abnormally clumped together A few capillaries containing red cells, traverse the gland, but the surrounding tissue is not vascularized The graft bone, seen below, has living osteocytes The osteogenic layer, particularly on the side near the gland, is disorganized The small perforation opposite the gland is not bordered by osteoclasts, and is probably a vessel foramen which was present already when the graft was made Haematoxylin Orange G Erythrosin Magn $\times 290$

PLATE 2

- Fig 9 Grey lethal parathyroid and bone grafted to a normal host Resorption accompanied by new bone formation is well marked opposite the gland, and this area contrasts with the thicker bone on the left hand side The cement-lines here demarcate the original bone graft from new deposits on both its surfaces The host skull is not resorbed Haematoxylin Orange G Erythrosin Magn $\times 80$
- Fig 10 Grey lethal parathyroid and bone grafted to a normal host To the left of the small parathyroid is a number of thyroid vesicles The bone graft is sharply perforated opposite the gland Haematoxylin Orange G Erythrosin Magn $\times 80$
- Fig 11 Normal parathyroid and bone grafted to a grey lethal host Parathyroid small with thyroid vesicles on left side Opposite the gland is a considerable area of reticulate new bone osteoclasts occur along its concave surface On either side the bone is thicker Several spicules, apparently the remains of the original graft bone, are situated close to the parathyroid Osteoclasts marked with ink, one of them is unusually large Haematoxylin Orange G Erythrosin Magn $\times 110$
- Fig 12 Grey lethal parathyroid and bone grafted to a grey lethal host The tissue around the parathyroid is heavily infiltrated The graft bone is uniformly thin A few large osteoclasts, marked with ink, occur on its concave surface Haematoxylin Orange G Erythrosin Magn $\times 110$
- Fig 13 Normal pituitary grafted with normal bone to a normal host A compact piece of anterior lobe tissue lies in close apposition to the bone graft which is neither resorbed nor markedly thickened in this position. At the extreme right hand edge of the bone graft there is a new deposit of spongy bone Haematoxylin Orange G Erythrosin Magn $\times 85$
- Fig 14 High power view of normal anterior lobe tissue attached to normal bone and grafted to a normal host At the lower part of the field is the spongy new bone with living osteocytes deposited on the bone graft surface Osteoblasts are seen along the surface of this new bone Compared with an ungrafted pituitary of similar age (inset) the cells of the grafted gland are seen to have many pyknotic nuclei Haematoxylin Orange G Erythrosin Magn $\times 380$

- Fig 15 Normal thyroid grafted with normal bone to a normal host. The thyroid follicles, which are widely scattered, are often distended with colloid. A thick layer of spongy bone has been deposited on the concave surface of the bone graft, and is demarcated from the original surface by a cement line. This new deposit is slightly thinner opposite the thyroid. There are no osteoclasts on the surface of the graft bone. The host skull is much thickened. Haematoxylin Orange G Erythrosin. Magn. $\times 85$
- Fig 16 Graft of capsular fragments of normal adrenal with normal bone to a normal host. Towards the centre of the field lies a piece of regenerated cortical tissue, showing a fascicular arrangement of cells. Both the bone graft and host skull are much thickened and are synostosed near the right hand side of the field. Haematoxylin Orange G Erythrosin. Magn. $\times 85$
- Fig 17 Graft of normal non ossifying costal cartilage with normal bone into a normal host. The cells of the cartilage are mainly alive except at the cut surfaces. Over the area covered by the cartilage the graft bone is thin, but osteoclasts are not found on its surface. On the left hand side the contrast between this thin region and the thickened bone to one side is very marked. Haematoxylin Orange G Erythrosin. Magn. $\times 85$





THE STOMACH IN SOUTH AFRICAN INSECTIVORA, WITH NOTES ON THE ORGANIZATION OF MAMMALIAN GASTRIC GLANDS

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As part of a systematic study of the Insectivora, a comparative anatomical investigation was initiated on three species readily available in South Africa the elephant 'shrew', *Elephantulus myurus*, the golden 'mole', *Eremitalpa grandis*, and the shrew, *Suncus orangiae*. These three species belong to different suborders of the Insectivora: Macroscelidoidea, Chrysochloroidea and Soricoidae (Simpson, 1945).

In the course of this investigation attention was directed to a study of the morphology of the stomach, and it soon became apparent that the stomach in the three insectivores differs greatly in gross morphology. The stomach in *Elephantulus* is a relatively simple sac, almost identical in structure with that of *Tupaia* and the primates. In *Eremitalpa* it has more the shape of a triangular pyramid, and in *Suncus* the fundic dilation next to the oesophagus is succeeded by a long, constricted, tubular portion (Pl. 1, figs. 1-3). The question arose whether these differences reflect important differences in structural organization or are merely superimposed upon a basic morphological similarity.

In his general discourse on the comparative anatomy of the alimentary canal of mammals, Flower (1872) outlined the gross structure of the stomachs of some Insectivora, and indicated their great variation in shape, but he did not describe the microscopical features. Edelman (1889) recorded observations on the histology of the cardia in *Erinaceus* and *Talpa*. Somewhat more complete is the account given by Carrier (1893) of the stomach of the hedgehog, *Erinaceus*. He found a narrow zone of cardiac glands, a fundic region extending to the middle of the greater curvature and bend of the lesser curvature, and a rather smaller pyloric region. Pernkopf & Lehner (1937) and Pernkopf (1937) noted that in *Sorex*, and even more in *Hydrosorex*, the pyloric region is drawn out and tubular, much as observed here in the related shrew *Suncus*. In the present study, the stomachs of *Elephantulus*, *Eremitalpa* and *Suncus* were examined in detail microscopically. Serial sections were prepared to define the different areas of glandular epithelium, and cytological techniques were employed to examine the structure and relations of the several cellular elements. As will be seen, in spite of structural differences the stomach in these three species is organized on a similar plan. This organization is simple, and serves to demonstrate with almost diagrammatic clearness the primitive interrelations of the different regions in the mammalian stomach, more or less undisturbed

by secondary specializations or adaptations. From examination of the arrangement in Insectivora it is thus possible to make some observations concerning the general problem of gastric organization.

MATERIAL AND METHODS

The stomachs used in this study were obtained from a number of adult male and female animals of each species, fixed in Bouin's fluid and preserved in 70 % alcohol or 10 % formalin. Portions of stomachs taken from *Elephantulus* and fixed in Zenker-formol were available for more detailed study. One stomach of each species was embedded in paraffin, sectioned serially and stained with haematoxylin and eosin in order to determine the distribution of the gastric glands. Further smaller blocks of stomach wall were embedded in paraffin and sectioned at 5 or 6 μ , these sections were stained to show the cytological features of the gastric epithelium. Of the staining techniques employed, Mallory's stain, Heidenhain's iron haematoxylin and mucicarmum and thionin were most satisfactory. The polychrome method of Giemsa, in which sections are overstained and differentiated in acidulated alcohol or colophonium, gave a very delicate demonstration of the structural features in the cells of the fundic glands, showing clearly chromidial substance as well as secretory products. Masson's silver impregnation was also employed, but no basal granular cells were detected in any of the stomachs investigated.

OBSERVATIONS

Elephantulus myurus *Gross morphology* The stomach (Pl 1, fig 3) is almost identical in structure with that of the lesser primates, having the form of an elongated, comma-shaped sac. The blind sac of the fundus passes over without any marked external change into the pyloric antrum, and thus, in turn, is separated by a shallow sulcus intermedius from the pyloric canal. The region of the pyloric sphincter is marked externally by a pyloric constriction. The lesser curvature between oesophagus and duodenum is short and has a definite incisura angularis. Internally, the gastric mucosa is gathered in a longitudinal direction. The muscular layer is thin over the greater part of the stomach wall, it becomes thickened in the pyloric canal, and at the pyloric orifice forms a well-defined sphincter (Pl 1, fig 6).

Macroscopical structure The peritoneal investment of the stomach is separated by a very thin layer of loose connective tissue from the underlying muscular coat.

The *tunica muscularis* consists of two layers. In the outer layer the muscle fibres are longitudinally disposed and continuous with the outermost coats of muscle of the oesophagus on the one hand and the duodenum on the other, the inner coat has circularly arranged fibres. The inner layer is about three times as thick as the outer over the fundus and becomes greatly hypertrophied at the pyloric extremity to form the pyloric sphincter. There is no oblique layer of muscle such as is usually found in the mammalian stomach. The *muscularis mucosae* forms a thin but well-defined sheet throughout the organ, but is not

particularly developed in any one region. It is interrupted wherever blood vessels penetrate into the tunica propria. Slight bands of smooth muscle extend longitudinally with these blood vessels deep into the mucosa, where they lie between the glandular tubules. The *tunica propria of the mucosa* is composed of loose reticular connective tissue. It forms for the most part a thin layer, but is thickened in some regions, and sends vascular projections into the larger folds of epithelium. These thickened regions of the tunica propria have usually a central blood vessel. There is no membrane of Zeissl or stratum compactum of dense fibrous connective tissue in the tunica propria as is found, for instance, in the cat.

The *epithelium of the mucosa* shows depressions or foveolae into which the gastric glands open. The zones of mucosa, arranged according to the type of glands in each, are indicated in Pl 1, fig 6. There are no oesophageal glands near the junctional region. The squamous stratified epithelium of the oesophagus is replaced by a narrow zone of *cardiac glands*, 2-3 mm in width. This zone is followed by a much greater *area of fundus glands*, which passes over very gradually into the *pyloric zone*. As the glands are traced from the fundus to the pylorus (Pl 2, figs 7-9), the chief cells become gradually less common and eventually disappear entirely. Parietal cells persist along with mucoid cells for some distance, however, in what is here termed the *transitional region*. The parietal cells themselves decrease in number at the base of the glandular crypts as the pylorus is approached, and are ultimately replaced entirely by mucoid cells of the *pyloric glands*. The cardiac and fundic glands are for the most part simple and tubular. The pyloric glands form a somewhat thinner layer, with glandular tubules branching and coiling more freely.

Cytology of the gastric epithelium. Surface cells. These cells cover the surface and pits of the mucosa. They vary considerably in size and shape, being adapted to the type of folding of the mucous membrane in each region. They have typically rounded or oval basal nuclei, with coarse chromatin blocks applied to the distinct nuclear membrane, and one or more plasmosomes. In mature cells the nucleus becomes smaller and stains more deeply. The luminal poles of surface cells have usually exceptionally well-preserved aggregations of fine granules of mucinogen secretion (Pl 2, fig 11, Pl 3, fig 14). Those cells deep in the foveolae have few granules, progressively more granules are seen towards the surface. The prosecretory granules stain intensely with iron haematoxylin, with acid fuchsin and the eosin component of Giemsa's stain. Eventually, they ripen to produce a typical theca at the apex of the cell, filled with mucin which stains deeply with mucicarmine. Such mature surface cells are rare in *Elephantulus*, they appear very similar to, though rather taller than, the surface cells of *Eremitalpa* shown in Pl 2, fig 12, and Pl 3, fig 15. At the junctional region between surface cells and mucoid cells numerous mitotic figures may be distinguished.

Mucoid cells. These are found in the cardiac and pyloric glands and the necks of the fundus glands. They vary somewhat in shape in different situations,

but are typically columnar, with rounded or oval basal nuclei. The chromatin again appears in masses, tending to be applied to the nuclear membrane. Plasmosomes are usually present. The cytoplasm of some of these cells is more or less homogenous, but most mucoid cells show varying degrees of accumulation of mucin secretion towards the luminal pole. This stains deeply with mucicarmine and metachromatically with thionin. With Mallory's stain the cytoplasm appears vacuolated, with strands of material coloured light blue (Pl 3, fig 18). Intermediate forms between mucoid cells and chief and parietal cells undoubtedly occur. Pl 3 fig 20, shows a cell containing a mucoid type of cytoplasm towards the lumen and typical parietal cell granules towards the base. The cell membranes are usually distinct, and there is little possibility of mistaken identification resulting from superimposition pictures. Other cells of mucoid type show accumulation of serous secretory granules. These are refractile and in some cases stain only faintly, in other cases they show increasing degrees of intensity of staining until they are indistinguishable from typical granules in chief cells. Coincident with this accumulation of serous secretory granules is an increasing basophilia of the basal pole of the cells (Pl 3, fig 19). Intermediate types of cell appear to be confined to the necks of the fundic glands, and occur not infrequently in the transitional region of mucous membrane between fundus and pylorus.

Parietal cells These are most commonly found in the upper third of the fundus glands, where they have a characteristic position somewhat removed from the lumina of the tubules and related to the bases of mucoid and chief cells (Pl 3, figs 17, 18). The parietal cells are triangular or rounded in shape. They have large, rounded, central nuclei, fairly coarse chromatin, and usually one or more plasmosomes. Binucleate cells are not uncommon. The entire cytoplasm is filled with secretion, but for small spaces or 'canals' in the region of the Golgi apparatus. The secretion itself occurs as discrete subangular granules, not highly refractile, staining purple with Mallory's stain and reddish with Giemsa's preparation. Parietal cells near the necks of fundic glands tend to be smaller than those deeper in the tubules. In some parietal cells granules staining like those in chief cells occur. Very occasionally these granules may accumulate in such numbers as to distort the cell and make it difficult to decide whether it is, in fact, a parietal cell at all. Near the bases of the glands some small, rounded, vesicular parietal cells free of granules may be observed. Some of these cells have pyknotic nuclei and appear to be undergoing degeneration. As the chief cells become fewer and disappear towards the pylorus, the parietal cells are found nearer to, and ultimately at, the base of the glands. Parietal cells become first more and then less numerous as one passes through the transitional region, and are ultimately replaced entirely by mucoid cells. As has been noted, cell types intermediate between mucoid and parietal cells occur.

Chief cells These are columnar elements found only towards the base of the fundic glands. A typical chief cell has a large, rounded or oval nucleus basally situated, with irregular blocks of chromatin (Pl 3, fig 17). The area of cyto-

plasm between the nucleus and basement membrane contains diffuse chromidial substance, which is stained light blue in sections prepared with Mallory's technique, and more deeply by toluidin blue. The luminal pole of the cell is filled with a mass of spherical, refractile secretory granules, varying somewhat in size, and staining with the orange-G component of Mallory's mixture and purple with the technique of Giemsa. At the bases of glandular tubules, a number of chief cells are found with very large refractile granules distributed throughout the cytoplasm. There is occasionally evidence of nuclear karyorrhexis and karyolysis, suggesting that some of these cells degenerate and are cast off in the lumina of the glandular tubules. Here again, types intermediate between chief cells and mucoid cells are found (Pl 3, fig 19).

Suncus orangiae *Gross morphology* In different specimens of *Suncus* the stomach was found to vary somewhat in size and shape. The organ has typically a large fundic dilatation, which is delimited by a slight constriction from the succeeding long, thin, tube-like region. At first sight this tubular portion of stomach appears to be the most proximal part of the duodenum, but closer inspection reveals a slight pyloric constriction some 5 mm along its length, and this marks the transitional region between the two organs. When the stomach is cut, its wall is seen to be unusually thin. The muscular coat, poorly developed over the greater part of the organ, becomes slightly increased as the pyloric extremity is reached, and here forms a small pyloric sphincter (Pl 1, fig 5). The gastric mucosa is likewise relatively thin, and is less folded than in either *Elephantulus* or *Eremitalpa*.

Minute structure There is very little connective tissue between the simple squamous *peritoneal mesothelium* and the subjacent tunica muscularis. The tunica muscularis itself, as has been noted above, forms a thin bilaminar sheet. The longitudinal and circular layers are of roughly equal thickness over the most part of the organ, but the latter becomes increased to form a sphincter at the pyloric extremity. The inner layer is characteristically folded or puckered in cross section in fixed preparations.

Four main regions of *mucosa*, each having glands of characteristic type, may be distinguished (Pl 1, fig 5). The squamous stratified epithelium of the oesophagus is succeeded by a narrow *zone of cardiac glands*, some 3 mm wide. At the edge of the cardia is a narrow zone (not figured) with glands composed of parietal as well as mucoid cells. Farther from the cardia, chief cells become rapidly more abundant until, in the true *fundus*, they make up the lower half of each gland. As the limit of the fundic region is approached, chief cells diminish in number, and parietal cells occur in the lower third of glandular tubules. In the *transitional region* there are no chief cells, although many mucoid cells near the fundus show slight basophilia and other signs of differentiation, parietal cells are found in the basal part of the glands, and mucoid cells make up the rest of the glandular epithelium. Parietal cells diminish in number as the true pyloric region is reached, and the glands of the *pylorus* have only mucoid cells.

Cytology of the epithelium No discrete granules of secretion occur in *surface cells*, nor is there an intense mucin reaction. The luminal pole of many surface epithelial cells does stain to some extent with mucicarmine, however, and this may be taken as evidence that the cells are mucin secreting (Pl 3, fig 16). Mitoses in the gastric glands again appear to be confined to the junctional region between surface cells and mucoid cells. The mucoid cells, chief cells and parietal cells have a cytology and distribution much as in *Elephantulus*.

Eremitalpa granti *Gross morphology* The fundic region of the stomach is relatively large and dilated (Pl 1, fig 1), and forms a well-defined sac which has roughly the shape of a triangular pyramid. This is succeeded by a narrow pyloric region which passes over into the duodenum, the junctional area being marked externally by a distinct pyloric constriction. The muscular layer is only moderately developed over the most part of the organ. At the pyloric extremity it becomes progressively thickened until it forms a sphincter surrounding the pyloric orifice (Pl 1, fig 4). The mucosa is slightly rugose.

Microscopical structure As in the other insectivores, the *tunica muscularis* has only two layers, the outer more or less longitudinally orientated, the inner circular. The inner layer is approximately twice as thick as the outer over the greater part of the organ, and considerably more so towards the pyloric extremity and sphincter.

The four regions of *mucosa*, already described, each having glands of characteristic type, may be distinguished (Pl 1, fig 4). The stratified epithelium of the oesophagus is replaced immediately by a *zone of cardiac glands* some 4 mm in width. Cardiac glands are simple and tubular, and made up entirely of mucoid cells. At the edge of the cardia, glands are found containing, in addition to the typical mucoid cells, some parietal cells. As the *fundus* is reached, parietal cells become abundant and chief cells appear in increasing numbers at the base of the glands. In the body of the fundus, the simple tubular glands have a fairly regular arrangement of cells. From the blind end to roughly half-way along each gland almost exclusively chief cells are encountered, parietal cells predominate in the succeeding region, and pass over into an area with mostly mucoid cells near the neck. Towards the pylorus the chief cells at the base of the glandular tubules become less abundant, and their place is taken by parietal cells in the *transitional region*. In this zone mucoid cells gradually replace the parietal cells, and in the *pylorus* mucoid cells occur alone.

Cytology of the epithelium Surface cells in *Eremitalpa* are typically rather low columnar elements, with oval, basally placed nuclei, distinct chromatin blocks and plasmosomes, and have almost invariably a luminal theca filled with mucus (Pl 2, fig 12, Pl 3, fig 15). Granules of pre-mucin are never seen, the theca appears as a faintly granulated or vacuolated mass staining intensely with mucicarmine. As in *Elephantulus* and *Suncus*, most mitotic figures are confined to the junctional region between mucoid cells and surface cells. The cytology and distribution of chief and parietal cells are almost identical with those already described in *Elephantulus*.

DISCUSSION

General features The stomachs of the three Insectivora studied—*Elephantulus*, *Suncus* and *Eremitalpa*—show considerable differences in shape and size (Pl 1, figs 1–3), yet the minute structure of each is very similar (Pl 1, figs 4–6), rather closely resembling that previously described in *Erinaceus* and *Talpa* (Pernkopf & Lechner 1937). In each case, the stomach has only longitudinal and circular coats of muscle, a narrow zone of cardiac glands some 3–4 mm in width, which is gradually replaced by true fundic glands, and a broad transitional region between fundus and pylorus. The principle of organization is the same in all cases, and it is suggested that the arrangement here described in the Insectivora may be taken as representing a primitive and generalized mammalian pattern. Although the different species studied are widely different systematically and each is adapted to a different mode of life, the stomach structure does not show any important differences. Apparently, the cornified epithelium and other specialized features which are found, for instance, in the stomachs of rodents, edentates and ungulates, do not occur in insectivores.

Gastric mucins The precise part in gastric physiology played by the various cells in the epithelium of the mucous membrane is still obscure. Even the histochemical demonstration that chief cells in the gastric glands secrete the enzyme pepsin, while parietal cells are acid-forming, is not by any means satisfactory. As far as the mucin-secreting cells are concerned, although all their products stain with standard dyes like mucicarmine or mucihaematin, there is little doubt that they differ one from another, a surface cell being as different from a mucoid cell as either is from a goblet cell or a secretory cell of Brunner's glands. This difference is not only confined to their morphology, but applies also to the details of their mode of secretion, although those cells near the zone of mitoses between glands and foveolae are not fully differentiated, as Zanotti (1941) emphasizes. Hence, it is likely that the mucin secreted is in each case specific, even though giving the same general reactions. It is well known that the mucins found in different cells vary in their affinities for specific stains like mucicarmine, but at present no further means of differentiating these related chemical substances exists.

The surface cells of the stomach show some differences in the various species of Insectivora. In *Elephantulus* most of them, irrespective of whether the material had been fixed in Bouin's fluid or Zenker formol, show unusually well-preserved granules of mucinogen in the luminal poles (Pl 2, fig 11, Pl 3, fig 14). Sometimes this prosecretion can be seen ripening and giving the typical mucous reaction, so that the cells are undoubtedly mucin secreting. It is noteworthy, however, that such prosecretory granules were never observed in either of the other insectivores, nor do they appear to have been described in other mammals. Whether this difference in mode of elaboration of mucin in *Elephantulus* indicates an actual difference in the ultimate secretory product is impossible to decide with available techniques.

The transitional region It is common in the literature to find descriptions of the junction between fundus and pylorus as abrupt. However, in each of the insectivores examined the fundo-pyloric transition is gradual. In the fundus the cells are arranged in fairly distinct layers. Chief cells are almost exclusively found near the blind ends of the glandular tubules, they are gradually replaced by parietal cells, and the latter, in turn, pass over into a zone of mucoid cells adjoining the surface cells of the gastric pits. As the edge of the fundus is approached, chief cells become less numerous and parietal cells are found farther down the tubules. Parietal cells persist along with mucoid cells for a considerable distance before the true pylorus (the glands of which comprise only mucoid cells) is reached. This area of parietal and mucoid cells (Pl 2, fig 8) forms a true transitional region. Such a transitional region is by no means confined to the Insectivora, although it appears to reach its greatest development in this group. Lim (1922) found in the cat an area of pylorus in which small parietal cells were present, Aschoff (1924) was able to write 'The most recent researches of Oshikawa have, however, shown, as Ellenberger had previously demonstrated for the dog, that there is still a third form of gland which should receive the appellation of intermediary glands. These are glands which almost entirely lack chief cells but which, on the other hand, contain parietal cells in varying numbers.' In human (Bantu) material, Gillman (1939) found a transitional zone such as that described above, and a similar persistence of parietal cells along with mucoid cells has been noted in *Orycteropus* (Allison, 1947).

It is clear, then, that a transitional region has been demonstrated between the fundus and pylorus of widely different species of mammals, and it is likely to be found on more careful observation of the actual fundo-pyloric transition in other species. It should, therefore, be regarded as an essential feature of gastric structure. A similar, but far narrower, transitional zone occurs between fundus and cardia.

The gradient of organization in the gastric glands, and the degree of differentiation of their epithelial cell types If the arrangement of cells in the gastric glands of Insectivora is diagrammatically indicated, as in Pl 3, fig 13, two features are apparent. In the first place, the mucoid neck cells in the fundus appear as a thin cellular sheet overlying the other epithelial elements of the fundus and connecting the cardia and pylorus, the cells of which are identical morphologically and, presumably, physiologically. In the second place, the layers of different cells exhibit an orderly arrangement. It is evident that there are here three lines of gradient, each originating from the base of the fundus glands: one passing along the cardia towards the oesophageal epithelium, another along the glandular tubules towards the surface epithelium, and a third along the transitional region and pylorus towards the duodenal epithelium. Each of these gradients shows essentially the same features: a zone of chief cells, gradually replaced by parietal cells, and these, in turn, replaced by mucoid cells. In other species such as the cat (Lim, 1922), additional features fit into

the same gradient. Thus, the parietal cells nearest the chief cells are large, while smaller parietal cells are found with mucoid cells in the necks of fundic glands and in the glands of the transitional region. Moreover, those mucoid glands of both pylorus and cardia farthest away from the fundus and nearest the duodenum and oesophagus respectively are excessively coiled. This appears to be the fundamental gradient of organization in the gastric glands. The gradient begins with chief cells and ends with mucoid cells, and it is possible to relate it to the differentiation of the cells in the gastric glands.

It has long been held that of the gastric glandular elements chief cells are most differentiated and mucoid cells least differentiated. Evidence in support of this view may be adduced from many sources. Lim found that the first cells to differentiate in embryonic gastric glands are mucoid, from these mucoid cells first parietal and later chief cells develop. Harvey (1907) performed gastro-enterostomies on dogs and studied cell changes along the line of suture, he found chief cells for a distance of 7 mm. changed to mucoid cells which persisted for periods up to 3 weeks, and which afterwards were retransformed to chief cells. When the pylorus was occluded also, pyloric glands did not show any tendency to assume the characters of fundus glands. Cade (1901) had earlier observed that parietal cells disappear and chief cells lose their granules in the vicinity of gastro-enterostomy openings in dogs, and all the cells appear mucoid in character. He inferred that the altered conditions had caused the specialized chief cells to revert to the more primitive mucoid cells. Lim confirmed Cade's findings in cats and agreed with his conclusions.

Ferguson (1928), studying regeneration after removal of 25 sq cm. of gastric mucosa in dogs, was of the opinion that 'the evidence indicates that the specialized cells of the gastric glands are capable of passing through a cycle of transformations in response to injury followed by regeneration. Thus they are first dedifferentiated into more primitive cells which multiply and migrate out to cover the denuded surface. These foveolar cells then increase by mitosis and form gland *anlagen*. Following this, the cells are transformed into the specialized cells from which they arose and the cycle is completed.' Mucoid cells are not mentioned, and it is likely that those foveolar cells from which chief and parietal cells developed were, in fact, mucoid. Moreover, displaced gastric glands—such as are found in oesophageal 'erosions'—are almost invariably of mucoid, i.e. least differentiated, type.

In normal adult stomachs mitoses are frequent in the neck region of fundic glands. Although most of the cells resulting from these mitoses seem to replace surface cells, some mucoid cells are constantly formed, and these appear to differentiate into both chief and parietal cells. The origin of the chief cell from the mucoid neck cell has been repeatedly postulated in the German literature (Plenk, 1932). All stages of transition between the two cell types may be found in the necks of fundic glands. A similar origin of parietal cells from mucoid cells in the adult appears to have been overlooked, even though the ontogenetic and experimental studies of Lim and Ferguson cited above have shown that

under certain conditions mucoid cells may be transformed into parietal cells. Satisfactory descriptions and figures of intermediate cell types are rare, indeed, most authorities, like Bensley (1932), describe abrupt replacement of neck cells with no intermediate cell types. However, Gillman (1939) has observed transitional forms between mucoid cells and parietal cells in the necks of fundic glands and the glands of the transitional region, and it was possible in this study to identify cells intermediate between mucoid cells and both chief and parietal cells. Mucoid cells appear to have, then, a prospective potency to transform into chief and parietal cells. Whether or not this transformation is achieved depends upon the environment of the cell. The intermediate cell types are always in the same location—either in the necks of the fundic glands or in the transitional region between fundus and pylorus or cardia; this means that they are found in the junctional region between cell zones, which might account for their partial differentiation.

The occurrence of mitoses in the neck region of the glands, the evidence that cells differentiate from mucoid to chief and parietal cells, and the presence of apparently degenerative elements near the glandular bases, all together suggest that chief and parietal cells in the adult ultimately 'wear out', are cast off into the lumina of the glands and replaced from above. This implies a gradual migration of cells down the glandular tubules and a less static and more free relationship between epithelial cells and subjacent supporting tissue than is generally envisaged.

The place of parietal cells within this scheme is not quite clear. Parietal cells can differentiate from mucoid cells in the embryo and adult. They are most common in the luminal half of the fundic glandular tubules, but they can be found not infrequently more basally situated among the chief cells. This seems to indicate that the specialization of parietal cells involves some restriction of possible development, so that, even though parietal cells are found in a chief cell area, they are not as a rule transformed into chief cells. This developmental restriction is clearly but a temporary one, for Cade and Lam found parietal cells under certain experimental conditions dedifferentiating into cells of mucoid type, which are pluripotent. In the adult stomach there is, indeed, sometimes to be found cytological evidence suggestive of direct transition between parietal and chief cells.

All the evidence cited above supports the view that the cardia and pylorus are relatively undifferentiated areas of stomach. It seems more correct to consider the cardia in this way as undifferentiated rather than to conclude with Bensley (1902) that differentiation of the cardiac mucous membrane may be effected by the action of mechanical forces naturally produced, operating through many successive generations, and associated with natural selection. In keeping with the view that the fundus is a relatively highly differentiated area is the observation that epithelium of intestinal type, when it occurs in the stomach, is usually confined to the pylorus.

Organization of the stomach. It seems that at least two factors control the

morphological organization of the stomach. One, perhaps acting primarily on the outer coats of the stomach wall, controls the general shape and disposition of the organ. The second controls the differentiation of the glandular epithelium, and appears to act primarily from the base of the fundic glands. In the Insectivora it is apparent that these factors can act independently, since the general shape of the organ may vary greatly without appreciably affecting the intrinsic organization. However, our knowledge of these tertiary grade organizer phenomena—particularly those at work in adult tissues—is insufficient to allow of many generalizations regarding the means by which differentiation is brought about in the living organism.

SUMMARY

1 The gross morphology and minute structure of the stomach in the unrelated South African insectivores, *Elephantulus myurus*, *Suneus orangiae* and *Eremitalpa granti*, are described in detail. The stomachs exhibit considerable variation in gross structure, but histological study shows that in each species the stomach is built on the same pattern of organization. In each case there are only longitudinal and circular layers of the tunica muscularis, a narrow cardia and, between the major fundic and pyloric areas, a wide transitional region having parietal as well as mucoid cells.

2 The cytology of the mucoid, chief and parietal cells is much the same as in other mammalian species. The surface cells in the different animals studied show structural variations suggesting species differences in the mode of elaboration of their mucous secretory products. It is suggested, further, that, since surface and mucoid cells differ in their cytology, the character of the mucins they produce is probably also different.

3 The stomach in insectivores shows a generalized arrangement of glandular cells, in which it is possible to establish a gradient of organization passing from the base of the fundic glandular tubules towards the oesophagus, the surface of the stomach and the duodenum respectively. These lines of gradient have all essentially the same features—chief cells replaced by parietal cells and these, in turn, replaced by mucoid cells. This gradient is discussed in relation to the concept that the chief cells are the most differentiated of the gastric glandular elements, and the mucoid cells the least differentiated. It is suggested that in the adult stomach, as in the foetus, mucoid cells as well as surface cells are formed by mitoses in the necks of fundic glands, and that some of these are transformed into chief and parietal cells. Intermediate cell types found in the neck zone of fundic glands and in the transitional region are described and illustrated.

4 It seems possible to distinguish two factors which control the organization of the stomach: one acts upon the general shape and disposition of the organ, the other influences the differentiation of the glandular epithelium. These factors work independently to produce the stomach pattern characteristic of each species.

My thanks are due to Prof R A Dart, in whose department in Johannesburg most of the work was carried out, and to Prof C J van der Horst, through whose courtesy the material was obtained. I am indebted to Dr J Gillman for helpful advice.

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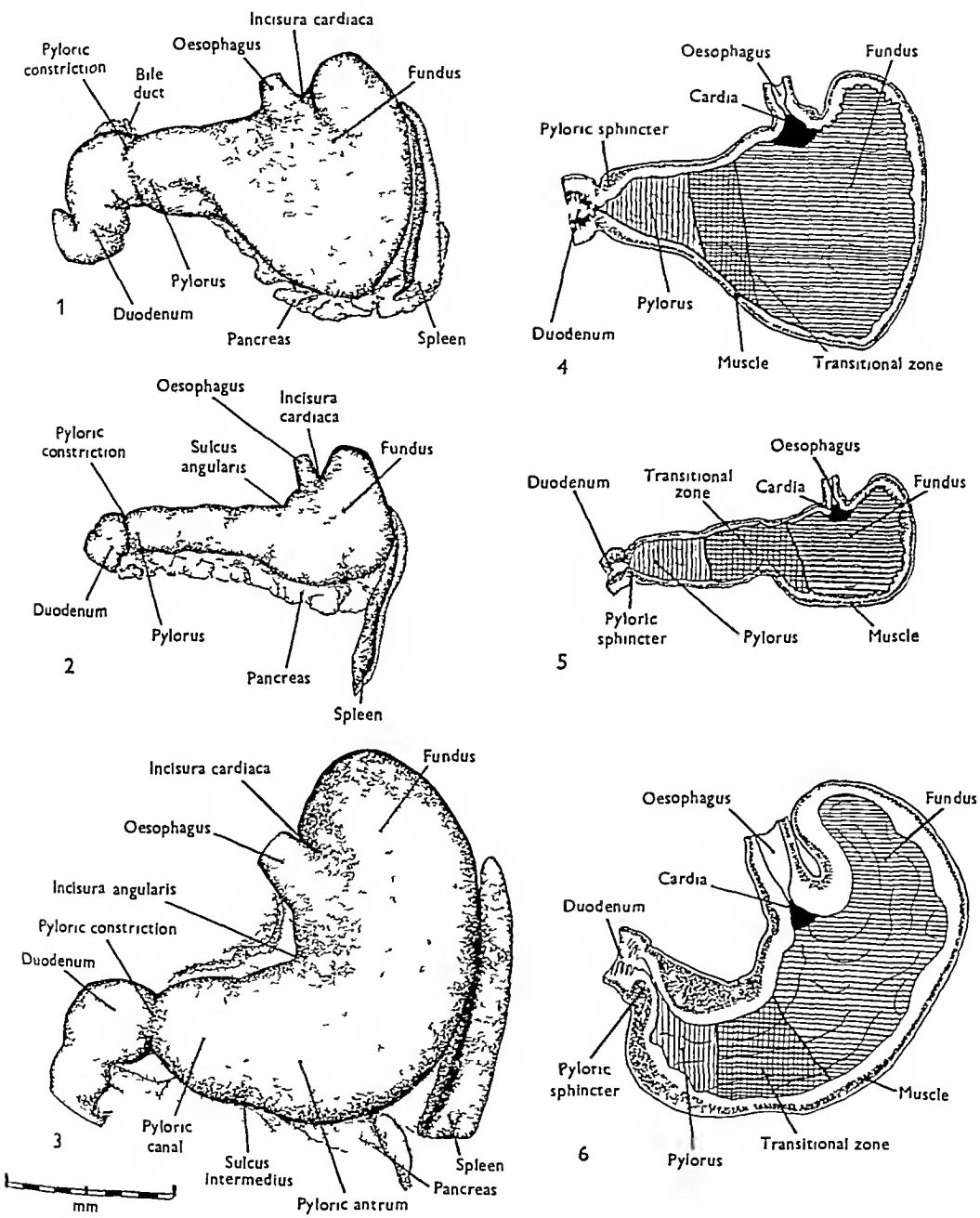
EXPLANATION OF PLATES

PLATE 1

- Figs 1-3 Drawings of the stomachs of *Eremitalpa*, *Suncus* and *Elephantulus* respectively
- Figs 4-6 Drawings of median sagittal sections through the stomachs of *Eremitalpa*, *Suncus* and *Elephantulus*, indicating the disposition of the muscular coat and the different regions of gastric mucous membrane

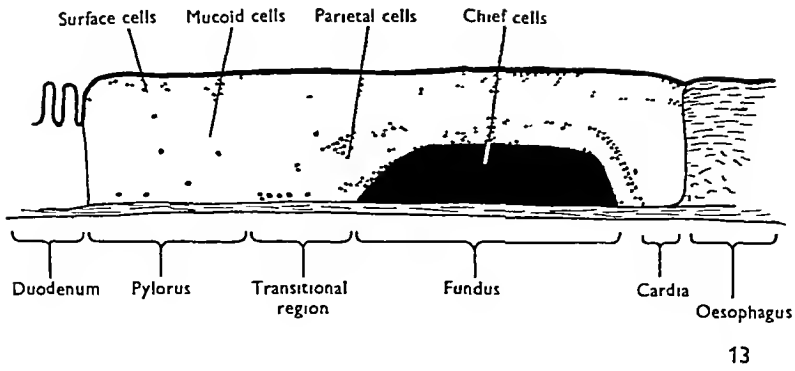
PLATE 2

- Fig 7 Glands of the fundus in *Elephantulus*. The deeply stained chief cells are seen towards the base of the glandular tubules. Zenker formol, haematoxylin and eosin $\times 250$
- Fig 8 Glands from the transitional region in *Elephantulus*, showing parietal and mucoid cells. Arrows indicate mitotic figures between glandular and surface epithelium. Zenker formol, haematoxylin and eosin $\times 250$





ALLISON—THE STOMACH IN SOUTH AFRICAN INSECTIVORA



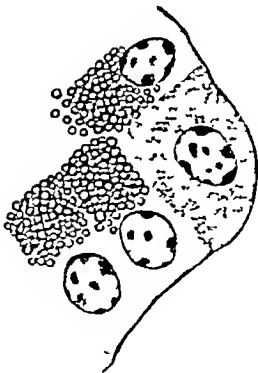
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- Fig 9 Pyloric epithelium of *Elephantulus*, showing foveolae and simple mucoid glands characteristic of both pylorus and cardia Zenker formol, haematoxylin and eosin $\times 200$
- Fig 10 The edge of the fundic region of *Elephantulus*, showing the deeply basophilic chief cells decreasing in number at the base of the glandular tubules towards the right of the field Parietal cells along with chief cells persist in the transitional region at the extreme right Zenker formol, haematoxylin and eosin $\times 70$
- Fig 11 Surface cells in *Elephantulus*, showing the accumulation of secretory granules in the apical portion of each cell Zenker formol, iron haematoxylin $\times 800$
- Fig 12 Surface cells in *Eremitalpa*, showing the intensely staining mucus in the apical theca of each cell Bouin, iron haematoxylin and mucicarmine $\times 800$

PLATE 3

- Fig 13 Diagrammatic longitudinal section through the gastric mucosa of an Insectivore, indicating the relationship of those areas in which the different cell types are predominant
- Fig 14 Surface cells in *Elephantulus* Bouin, iron haematoxylin $\times 800$
- Fig 15 Surface cells in *Eremitalpa* Bouin, iron haematoxylin and mucicarmine The theca stains intensely red $\times 800$
- Fig 16 Surface cells in *Suncus* Bouin, iron haematoxylin and mucicarmine The luminal pole of the pole stains diffusely reddish $\times 800$
- Fig 17 From a glandular tubule in *Elephantulus* Bouin, Mallory Refractile zymogen granules in chief cells stain orange, basal chromidial substance blue, granules in parietal cells purple $\times 800$
- Fig 18 From the neck of a glandular tubule in *Elephantulus* Bouin, Mallory Cytoplasm of mucoid cells blue $\times 800$
- Fig 19 A cell from the neck of a fundic glandular tubule in *Elephantulus*, intermediate in type between a true mucoid cell and a chief cell Bouin, Mallory Granules stain faintly orange, chromidial substance light blue $\times 800$
- Fig 20 A cell intermediate in type between a mucoid cell and a parietal cell Bouin, Mallory From the transitional region in *Elephantulus* $\times 800$

THE EFFECT OF PRESSURE ON NERVE FIBRES

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There is some evidence of fluid properties in the axoplasm of nerve fibres (Young, 1945), and the changes taking place in the myelin substance during degeneration suggest fluid behaviour of this also. The present work was undertaken to discover whether application of circumferential pressure to a section of nerve is able to displace axoplasm or myelin, as would be expected if these behave as liquids.

The effect of pressure on nerves has already been frequently investigated, experimentally and clinically, but most of the experimental observations have been concerned with the effects on conduction and the action potential. The methods of application of the pressure in previous investigations can be divided into two classes: (1) Those in which the nerve was cut and then threaded through some constricting mechanism. This method has been used by Meek & Leaper (1911), Erlanger & Gasser (1929) and Hodgkin (1937) in studies of action potentials. Weiss (1943) has adopted a similar principle in his studies of regeneration, using arterial sleeves for union of the severed nerve ends. (2) Those in which the nerve was left uncut and pressure applied laterally by (a) a wash-leather bag filled with mercury (Mitchell, 1872, Denny-Brown & Brenner, 1944), (b) a spring clip (Denny-Brown & Brenner, 1944), or (c) an air-inflated tambour (Bentley & Schlapp, 1943). These methods overcome the difficulties introduced by section of the nerve, but under such conditions the pressure is not evenly distributed round the circumference of the nerve and therefore the whole nerve bundle will tend to assume an oval shape.

Two distinct problems present themselves for solution: (1) To find a method of application of controlled circumferential pressure to a living nerve. (2) After application of the pressure to prepare specimens which bear a constant relation to the living fibre and from which it is practicable to make accurate measurements of the fibre diameters.

METHOD

The nerve to the medial head of the gastrocnemius (N.G.M.) of the rabbit was used. This is a small nerve bundle bound up with the tibial nerve, from which it can be dissected away to give an unbranched length of 3–4 cm. (Aitken, Sharman & Young, 1947). It contains some 400 medullated fibres, of which more than half have a diameter of over 10μ , the total diameter of the whole nerve is about 0.3 mm. The rabbits employed were healthy adults, but no attempt was made to use only one breed, weight or size of animal.

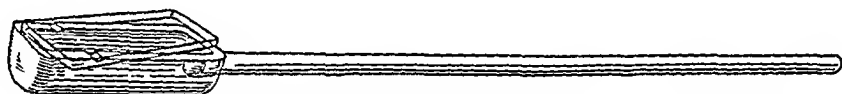
The pressure chamber consists of a glass trough fused on to the end of a glass tube (Text-fig. 1). The breadth of the trough is 1 cm., and on each side is

a carefully smoothed groove 1 mm deep. The chamber is closed by applying a glass lid which can be clamped on to it tightly by a rubber-covered screw clamp. The glass tube is connected by rubber tubing, on which there is fitted a control clip, to a mercury manometer.

Under nembutal and ether anaesthesia the *v. g. n.* is dissected carefully from the tibial nerve, and the latter is then cut across and drawn aside. The biceps muscle is divided across its fibres to provide easy access from the side, and to give two long muscle strips which can be laid over the chamber and clamped to minimize cooling and evaporation.

The possibility of applying a pressure in this way depends, of course, on finding a suitable seal to close the chamber where the nerve enters and leaves. Vaseline becomes fluid at body temperature. After trial of various other hydrocarbons and waxes success was achieved with the use of sodium alginate gel (kindly supplied by Alginate Industries, Ltd. on the suggestion of Sir T. Merton, F.R.S.). Made up with a small amount of water this substance will resist pressure up to one atmosphere.

The chamber, filled with mercury, was smeared round its upper edge with sodium alginate, and this gel was put along the edge of the under surface of the glass lid also. The chamber was slipped under the nerve from the side and



Text-fig. 1. Drawing of glass chamber with grooves in which the nerve is placed.

the latter was then manipulated so as to lie in the grooves, and the lid was put in place and firmly clamped. The nerve was placed at the bottom of the grooves to prevent adhesion to the glass cover, and the pressure was applied as quickly as possible after clamping down the lid, so that the mercury flowed between the glass and the nerve before the latter adhered to the glass. The mercury thus surrounded the nerve on all sides. The pressure employed varied between 0 and 670 mm. and was maintained for between 7 and 30 min.; the fixing fluid was then poured round the nerve and chamber whilst pressure was maintained for a further 5 min. The pressure chamber was then removed from the nerve, to allow access of fixative to the compressed region, and fixation was continued *in situ* for a further 25 min. The whole length of the nerve was finally removed in one piece and fixation completed by immersion in fixative for a further 24 hr.

FIXATION

The histological aspect of the problem is to obtain preparations that shall bear a constant relationship to the fibres in the unfixed state. Theoretically, any easily standardized technique would do, but in order to obtain useful measurements, the shrinkage and distortion must be reduced as far as possible; it would be difficult to obtain significant results from badly shrunk nerves in fixation.

as, for instance, with the use of Bodian's fixative or other silver technique. The condition of nerve fibres in the living nerve trunks is not certainly known, but from the appearances in freshly teased and in fixed preparations it is probable that the inner and outer edges of the myelin are perfect circles except near the nodes or near a Schwann nucleus. The technique in which they most nearly approach this form is fixation in Flemming's fluid, followed by embedding, sectioning and staining by the Weigert-Pal method (Gutmann & Sanders, 1943). A considerable proportion of the fibres appears round after this method (Pl 1, fig 1), nearly always, however, some of them assume an oval, or a polygonal or a crenated form. Moreover, at the centre of the bundle a few fibres often show other irregularities (Pl 1, fig 2) due to autolysis or some other change occurring before fixation is complete.

The fixative used was a variant of Flemming's fluid, consisting of 15 c.c. of 1% chromic acid, 4 c.c. of 2% osmic acid and one drop of glacial acetic acid (Sanders & Young, 1946). A solution containing 1 c.c. of glacial acetic acid in 20 c.c., as used by Flemming in his original solution (Baker, 1945), abolished all autolysis at the centre but gave marked crenation of all fibres. Complete absence of acetic acid, or less than one drop in 19 c.c., resulted in failure to penetrate to the centre of the bundle and, therefore, in much autolysis. The solution with one drop of acetic acid gave optimal results, but a further difficulty is that the solution is not very easy to standardize, and unless great care is taken varying results will be obtained. The drop of acetic acid was kept uniform by using a standard bore pipette. The osmic acid evaporates rapidly and diminution of the concentration of the osmic acid increases crenation. Nerves were examined which had been fixed for 24 hr. in Flemming's fluid that had been kept (1) in a carefully corked bottle sealed with wax, (2) in a corked bottle with no wax seal, (3) in a bottle with neither cork nor wax, (4) in an open Petri dish. Rapid evaporation of the osmic acid in conditions (3) and (4) led to both crenation and autolysis, but no difference could be seen between (1) and (2). When, therefore, the nerve was fixed *in situ* the hindlimb was carefully padded underneath so that the muscles formed an elongated trough filled with Flemming's fluid and the whole area was covered as far as possible to minimize evaporation. Fixation *in situ* only for 1 hr. was insufficient to give good staining of the centre of the nerve bundle.

After fixation the nerve was passed, without washing, through a series of alcohols, placed in cedar-wood oil for 24 hr., then placed for 15 min. in a mixture of equal parts of cedar-wood oil, benzole and paraffin, embedded in paraffin, and cut transversely at 5μ . Sections taken at 2 mm. intervals along the length of the nerve were stained by a modified Weigert method (Gutmann & Sanders, 1943). Differentiation was continued until the maximum contrast between the blue-black myelin and the background was obtained.

The amount of shrinkage produced by these procedures cannot be exactly estimated. Most of the shrinkage occurs during dehydration and embedding, and not during the actual fixation (Rexed, 1944, Baker, 1945). The aim

throughout the present experiments has been to keep the conditions under which the specimens were prepared absolutely uniform

The sections chosen for measurement were photographed directly on to bromide paper at a magnification of $\times 750$. By using a combination of a \pm mm objective with a $\times 8$ ocular, the whole nerve could be photographed on a single sheet of paper 29×24 cm. Magnification was checked before each set of photographs by photographing a micrometer scale.

Measurements All the myelinated fibres in each transverse section have been counted off into groups at 2μ intervals ($0-2$, $2-4\mu$, etc). While counting the photographs doubtful fibres were checked with the microscope. Small fibres with little myelin would otherwise be overlooked and the larger blood vessels, which stain slightly, might be mistaken for nerve fibres. For the assessment of diameter of the nerve fibres a series of circles of diameter $2\mu \times 750$, $4\mu \times 750$, etc., was accurately marked on a piece of perspex. Each nerve fibre, as it was allocated to its particular size group, was pricked on the photograph by a needle attached to a mechanical counter (designed by Dr V. Scotland). By matching the circles of known size with the nerve fibres, the size of the latter could be determined. In this way nerve fibres that were not perfectly circular could also be classified. All the sections in a given experiment were counted by the same person in order to eliminate the variation of assessment by different individuals.

The results of a count have been expressed as a fibre size distribution, and by the root mean square diameter (Sanders & Young, 1946) which will be referred to as D . This single figure gives an excellent guide to the relative size of the fibres in different sections, but must be used with care in the case of the *ngv* because of the marked bimodality of the distribution.

Controls Experiments were carried out to compare fibre number and fibre size (1) on the two sides of the same rabbit at three comparable levels, (2) at different levels in the same nerve, (3) at different levels in the same nerve when the nerve was put in the pressure chamber but no pressure applied.

Table 1 *The fibre number and root mean square diameter (D) at three levels of the left and right nerves to the medial head of gastrocnemius of four rabbits*

Rabbit no	Fibre no		Fibre no		Fibre no		D		D		D	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
742	268	281	272	284	263	291	13.0	12.3	12.7	12.2	12.7	12.6
743	362	359	352	368	358	363	11.4	11.0	11.1	11.0	11.1	11.0
744	353	355	352	350	351	369	12.3	12.1	12.5	11.9	12.5	12.2
745	381	375	380	377	385	377	11.4	11.3	11.4	11.4	11.2	11.7
	High		Mid		Low		High		Mid		Low	

Mean difference between sides (1) fibre no = 8, (2) $D = 0.3\mu$. Mean difference between levels (1) fibre no = 5.5, (2) $D = 0.17\mu$.

Table 1 shows the fibre number and D at three levels, high, mid and low in the left and right *ngv* of four untreated rabbits. The variation of fibre

number between opposite sides shows a mean of 8 fibres and the variation in D between opposite sides a mean of 0.3μ . Between different levels of the same side the mean difference of fibre number is 5.5 fibres and the mean difference of D , 0.17μ . In no case was the difference between the fibre distribution between levels significant. Eccles & Sherrington (1930) have shown that there is a change in the fibre number and size along the gastrocnemius medialis nerve in the cat, but their figures were based on sections over the whole length between 0.6 and 6 cm. from the muscle. The present experiments were carried out on 3 or 4 cm. of the N.G.M. The figures demonstrate that whilst there is a small but significant variation between the two sides, there is no variation either in fibre number or fibre size over the length of nerve used in these experiments, within the limits of accuracy of our determinations which are about 1.5 %.

Table 2 *The fibre number and root mean square diameter (D) at three levels of the nerve to the medial head of gastrocnemius from four rabbits. The nerve was put in a chamber with mercury and sealed but no pressure was applied*

Rabbit no	Fibre no	Fibre no	Fibre no	D	D	D
726	Above 356	At 360	Below 364	Above 12.5	At 12.6	Below 12.8
728	384	378	378	11.5	11.5	11.5
822	351	346	363	12.9	13.1	13.1
823	436	439	439	13.2	13.5	13.0

Mean difference between levels (1) fibre no = 5, (2) $D = 0.17\mu$

Table 2 shows the fibre number and D of four nerves which were put in the pressure chamber with sodium alginate seals, but without the application of pressure. The mean difference in the fibre number between levels is 5 fibres and the mean difference of D is 0.17μ . The difference in fibre distribution did not become significant at the 5 % level.

Results. Pl. 1 shows sections of two nerves (fixed and stained as described above) taken above, below, and at the site of pressure which in the case of no. 752 was 560 mm. Hg for 10 min. and in the case of no. 770 150 mm. for 10 min. At the site of pressure there was a marked diminution of the area contained within the perineurium. Planimetric measurement of the total area of the nerve bundle showed an average diminution of 28 % of the total area of the nerve bundle for all pressures of 80 mm. and over, and an average variation of only 4.6 % for pressures of 80 mm. or less.

A considerable proportion of this diminution in size of the whole nerve is in the tissues between the myelinated nerve fibres, the interstitial area. The tissues in this area are not stained by the Weigert technique except for the walls of the larger blood vessels. In addition to the blood vessels this interstitial area contains the endoneurial spaces and endoneurial fluid, the connective tissues and small unmyelinated nerve fibres. It seems probable that the marked diminution in this area is due to the expression of blood and endoneurial fluid.

At pressures above 80 mm the fibres tend to pack hexagonally, suggesting that the pressure outside is now greater than that within the fibres. The fibres at the edge of the nerve bundle tend to be more deformed than those at the centre, this is the behaviour to be expected of a number of narrow compressible tubes in a compressible envelope.

The actual size of the myelinated nerve fibres within the compressed region was compared with the size of the fibres at a similar level in an unoperated nerve of the opposite limb. In Table 3 the results of the experiments on 15 rabbits are tabulated, the pressure applied varying from 45 mm Hg to

Table 3 *The root mean square diameters (D) from three levels of nerves of fifteen rabbits after the nerve had been subjected to local pressure varying from 45 to 670 mm Hg, and from controls*

Rabbit no (1)	Pressure (mm Hg) (2)	Time (min) (3)	D above site of pressure (4)	D at site of pressure (5)	D below site of pressure (6)	D control (7)	D control minus D at site of pressure (8)	D above minus D at site of pressure (9)	D below minus D at site of pressure (10)	D below minus D above site of pressure (11)	χ^2 of col 11
823	45	10	13.2	13.2	13.5	13.2	0.0	0.0	+0.3	+0.3	17 (7)
794	50	10	11.2	11.7	11.1	12.2	+0.5	-0.5	-0.6	-0.1	5 (6)
822	75	10	13.0	12.6	12.7	13.0	+0.4	+0.4	+0.1	-0.3	6 (7)
538	80	10	11.1	9.5	11.6	11.2	+1.7	+1.6	+2.1	+0.5	77 (6)
795	100	10	11.7	10.7	11.8	11.7	+1.0	+1.0	+1.1	+0.1	11 (7)
770	150	10	10.8	9.8	11.2	10.5	+0.7	+1.0	+1.4	+0.4	13 (6)
737	250	10	13.1	11.3	13.4	11.4	+0.1	+1.8	+2.1	+0.3	12 (7)
721	300	10	12.5	11.7	13.0	12.9	+1.2	+0.8	+1.3	+0.5	27 (7)
667	370	30	12.5	11.5	13.0	11.9	+0.4	+1.0	+1.5	+0.5	18 (8)
578	475	7	12.0	11.3	12.2	12.3	+1.0	+0.7	+0.9	+0.2	22 (8)
709	500	8	14.5	13.0	14.0	13.9	+0.9	+1.5	+1.0	-0.5	16 (6)
710	500	8	12.4	11.3	12.6	12.1	+0.8	+1.1	+1.3	+0.2	27 (7)
752	560	15	12.0	10.5	12.0	11.6	+1.1	+1.5	+1.5	0.0	8 (7)
JS2	570	10	14.7	13.9	14.8	13.9	0.0	+0.8	+0.9	+0.1	38 (8)
586	670	10	13.7	11.7	14.4	12.7	+1.0	+2.0	+2.7	+0.7	39 (8)

670 mm Hg. The duration of application of pressure in the majority of cases was 10 min and no experiment has been included in which the pressure was maintained for 5 min or less. The root mean square diameter at the site of pressure and of the control nerve are shown in cols 5 and 7, and the difference between them in col 8. It will be seen that at pressures of 80 mm and over diminution of D was recorded at the site of pressure in all experiments except one. The anomalous experiment may be explained by the difference in D between nerves of the two sides, the average difference being 0.3μ . As has been shown above, comparison of the compressed region with other parts of the same nerve gives a more accurate interpretation. The mean difference between the compressed area and the control side is $0.80 \pm 0.44 \mu$.

Cols 9-11 of Table 3 show the differences in diameter along the compressed nerve. The fibres are smaller in the compressed region than they are either above or below (cols 9 and 10). To test the significance of these differences the hypothesis was set up that the frequency distribution of fibres in each size group was the same at the various levels. Application of a χ^2 test then showed

that there was a significant deviation from the hypothesis at the 1 % level when the fibre distributions above or below were compared with those at the site of pressure. By using a χ^2 test the difficulty of the statistical treatment of a bimodal distribution was overcome.

The differences in the diameter above and below the site of pressure (col 11) show that in ten out of twelve experiments with pressures of 80 mm and over, D was greater below than above the compressed region. In one case the measurements were equal and in one case the diameter below was less than that above. As before χ^2 was calculated for the distributions of fibre sizes above and below the site of pressure in each experiment, the values are shown in col 12 with the degrees of freedom in parentheses. Most of the individual values are significant on the 5 % level but not all. However, the total χ^2 is 292 with 79° of freedom for the experiments with pressures above 80 mm. Since the probability of finding such a high value of χ^2 is very low (less than 0.0001) we may conclude that the fibre sizes are larger below than above the area of pressure.

DISCUSSION

From the figures given it is clear that in the case of the nervus gastrocnemii medialis of the rabbit a pressure somewhere between 50 and 80 mm Hg begins to cause fluid to pass from the compressed part to the adjacent parts, both from the nerve fibres and the 'interstitial area'. Fluids moved by such small pressures cannot have a very great viscosity. As soon as pressures of 80 mm were reached the movement of the fluid in the fibres rose to a maximum and, except for individual variations, remained relatively constant up to 670 mm Hg.

Is it the myelin or axoplasm which is pushed out of the compressed fibres? Light is shed on this question by a consideration of the form of the myelin sheath in the compressed region. It will be seen that the myelin loses its circular outline when it is compressed, and assumes an outline which can be explained in part by the packing of the fibres, but an infolding of the myelin sheath also occurs. A possible explanation of these changes is that the myelin sheath, not being elastic, will fold rather than contract on the diminishing column which it has to contain. The myelin has not been measured separately, but there is no obvious change.

If the increase in fibre diameter above and below the site of pressure is due to axoplasm being expressed, then it is difficult to reconcile this movement with the presence of any histologically demonstrable membrane as suggested by von Muralt (1947).

The relative fibre size below and above the site of compression, shown in col 11, Table 3, points to a tendency for the expressed fluid to flow to the peripheral rather than to the proximal side. The differences are small, but cumulatively they are significant variations in the fibre diameters. They can be explained on the assumption that there is a small turgor pressure acting from the nerve cell body (Young, 1944) in a peripheral direction, that would tend to counter the pushing of the axoplasm toward the cell body.

SUMMARY

1 A method is described by which circumferential pressure can be applied to a nerve by immersing it in mercury and assessing the alterations in myelinated fibre size by measurement of photographs of transverse sections

2 No evidence of the tapering of the nerve fibres was found in the length of unbranched nerve used in these experiments

3 At the site of compression the whole nerve decreases in diameter on account of a large reduction in endoneurial fluid and a smaller reduction in the diameters of the nerve fibres

4 There is an increase in the diameter of fibres above and below the site of compression at pressures of 80 mm Hg, and over

5 The increase of diameter is greater below than above the compressed region, as would be expected if the cell body exercises a turgor pressure along the fibre

I wish to thank Prof J Z Young for his advice, and Dr Palmer for her assistance, Mr D Sholl for his advice on statistics, Mr F J Pittock and Mr J Armstrong for photographs and Mr J E Marner for technical help

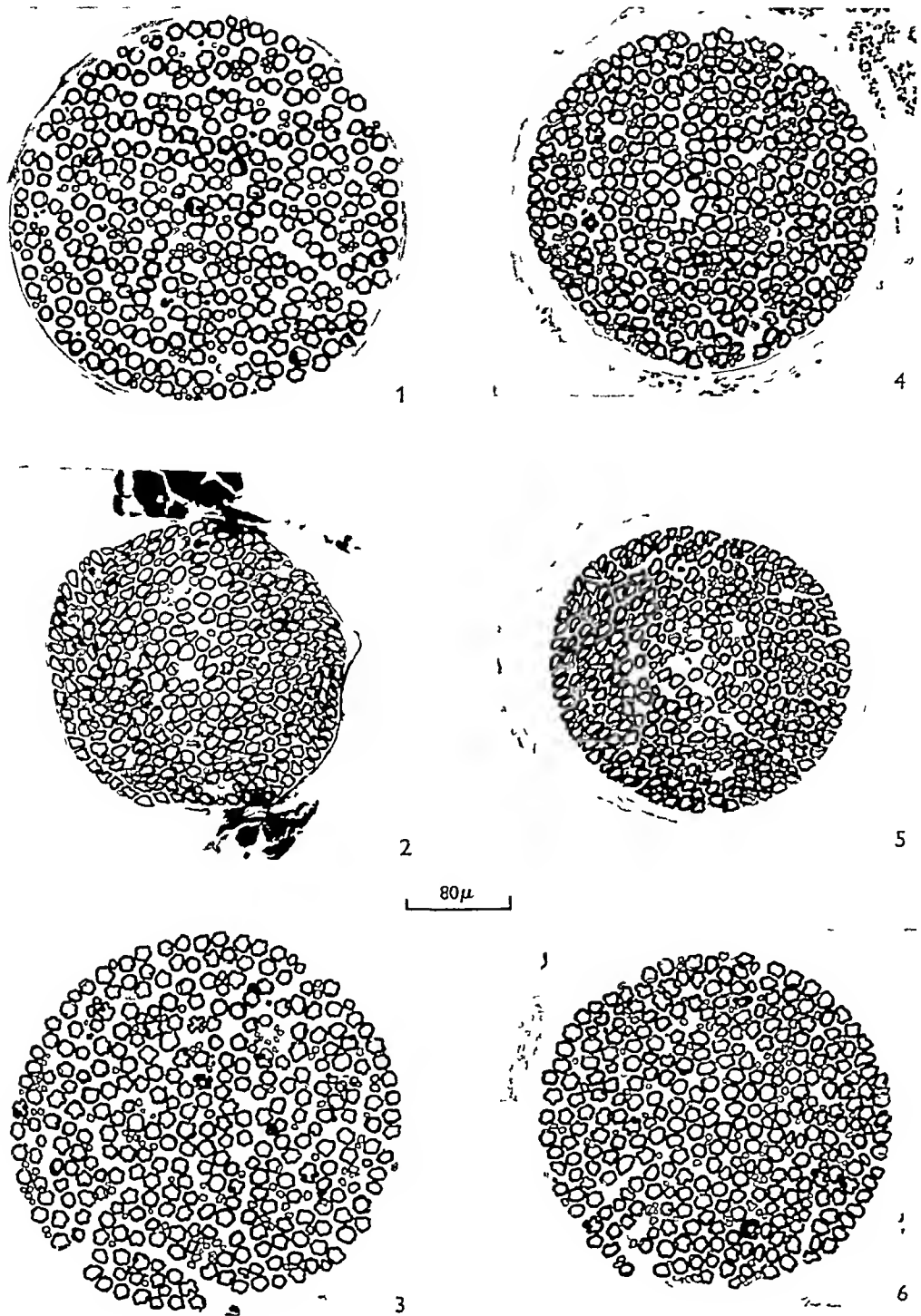
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EXPLANATION OF PLATE

All figures are of transverse sections of the nerve to the medial head of gastrocnemius of the rabbit, stained Weigert Pal stain

- Fig 1 Rabbit no 752 Section taken above the site of application of pressure of 560 mm Hg for 10 min
- Fig 2 Rabbit no 752 Section taken at the site of application of pressure of 560 mm for 10 min
- Fig 3 Rabbit no 752 Section taken below the site of application of pressure of 560 mm for 10 min
- Fig 4 Rabbit no 770 Section taken above the site of application of pressure of 150 mm Hg for 10 min
- Fig 5 Rabbit no 770 Section taken at the site of application of pressure of 150 mm for 10 min
- Fig 6 Rabbit no 770 Section taken below the site of application of pressure of 150 mm for 10 min



R E V I E W S

The Neocortex of Macaca mulatta By G VON BONIN and P BAILEY Illinois Monographs in the Medical Sciences, Vol v, No 4, 1947 (Pp 163, 62 plates \$ 3 00) Urbana, Ill The University of Illinois Press

This monograph deals principally with one aspect of the morphology of the cerebral cortex in *Macaca mulatta*, namely the cell laminae visible after staining with thionin. It is part of an extensive investigation into the comparative histology of the primate cerebral cortex, and, as such, is a valuable contribution to the problem despite the inevitable difficulties in interpretation which are partly technical and partly personal in origin.

On account of gross inaccuracies in Brodmann's publications the authors have reluctantly discarded his numerical notation in favour of Economo's less familiar symbols. They stress the absence of sharp dividing lines between the great majority of histologically separate areas, and produce a 'brain map' which eliminates many of the complexities existing in the literature. They mention, in particular, the presence of three distinct varieties of homotypical neocortex in the macaque, frontal, parieto-temporal and occipitotemporal, and are impressed by the great similarity in cortical organization between the macaque and man, suggesting that there may be a 'structural pattern of cortical organization' common to the primates and even to all mammals.

It is not quite clear why a chapter on the growth of the brain is included, especially as no details are given of the age or brain/body weight ratios of the monkeys used, and the question of variation in the pattern from brain to brain is not discussed. Similarly, it would have been valuable if the criteria adopted for establishing the identity of the various layers in different regions had been explained.

The work is particularly valuable in that it calls in question the generally accepted cortical maps and, by pointing out the inherent lack of correlation between this type of investigation and the physiological findings, stresses the need for further research. It is to be hoped that the other morphological characters will be taken into account as it is only in this way that the significance and homologies of the laminar pattern can be assessed.

The authors are to be congratulated on a large series of excellent photomicrographs which, together with a concise text, present the complicated subject in a form which is readily comprehended.

G J ROMANES

Organic Form and Related Biological Problems By SAMUEL J HOLMES (\$ 5 00) Berkeley and Los Angeles University of California Press 1948

In this volume Prof Holmes has put together twelve essays dealing with various aspects of the problem of organic form, with an introductory chapter which, only too briefly, summarizes the main arguments. Many of the essays have already appeared elsewhere and, as a result, there is a certain lack of integration in the volume as a whole—which can reasonably be regretted in contributions deeply concerned with regulating mechanisms. But if organic wholeness is lacking the volume does possess a continuous train of thought, mechanistic and strongly anti-teleological, relating to the problem of how organisms, and more particularly developing organisms, 'are able to regulate their activities so as to correct departures

from the normal course of living' If one is left, after reading the volume, with some sense of mental indigestion it is doubtless due to too much having been attempted in too little space Most of the chapters could easily justify expansion to full volume size Opinion, according to taste, knowledge and inclination, will naturally vary on the relative importance of the different chapters The reviewer found most enlightenment in the one called 'Genes *versus* Gradients' in which Child's views on gradients are subjected to a most convincing criticism The last chapter—'Recapitulation and its supposed causes'—is an interesting historical account of the part played by phylogenetic history in shaping the course of ontogeny

The most serious lack in Prof Holmes's approach to the problem concerned is, curiously, one common both to works, like the present, devoted to the mechanistic interpretation and to those with a markedly vitalistic bias That is, a refusal to face the problem of teleology as a whole Most contemporary biologists will be in complete agreement with Prof Holmes on what Santayana has so well called a 'mock explanation' Nevertheless, the teleology present in the world must be distinguished from final causes Woodger, whose statement of the problem is so cogent, but who is, surprisingly, never quoted by Holmes, has summarized the situation admirably (*Biological Principles*, p 451) 'the only real danger to be feared lies in being too easily satisfied with the belief that the last word has been said on this topic'

J D BOYD

OBITUARY NOTE

We have just heard with great regret of the death in Dublin of Professor J K Jamieson A notice *In Memoriam* will appear in our next issue

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